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METABOLIC HETEROGENEITY OF RIBONUCLEIC ACID IN EHRLICH ASCITES TUMOR CELLS[1)]

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INTRODUCTION

In studying the incorporation of ^{32}P-orthophosphate into nucleic acids of lymphatic cells obtained from a rabbit appendix, we have found that in the living cell there must be at least two species of ribonucleic acid (RNA) differing from each other in metabolic function: one of which is released by the treatment with aqueous phenol after Kirby's method, while the other can be extracted from the residue by Schmidt-Thannhauser's method. The specific activity of the former was found to be always very close to that of deoxyribonucleic acid (DNA), whereas that of the latter strikingly higher in activity (1). This has been confirmed also with normal and regenerating rat liver (I).

Indeed, of the greatest interest is the existence of the RNA fraction whose specific activity is closely similar to that of DNA. This fact leads us naturally to the supposition that this species of RNA, just like DNA in many cells, may be synthesized in rather strict relation to cell division, and, from a widely accepted view point of biological significance of RNA in protein synthetic processes, that in the course of cell division an important role may be played by this species of RNA.

Some results obtained with Ehrlich ascites tumor cells are reported in the present paper; it seems that the results support our supposition stated above.

MATERIAL AND METHODS

Ehrlich ascites tumor cells were transplanted into the abdominal cavity of hybrid albino mice that weighed about 20g. Seven or 10 days after inoculation, ^{32}P-orthophosphate (30μc/animal) was injected intraperitoneally, the animals being sacrificed 2 hours later to harvest the tumor cells. Recovered cells were washed once with cold 0.14M NaCl and hemogenized as usual.

An aliquot was used for the extraction of 'total RNA' by Schmidt-Thannhauser's method (2). The remainder of the homogenate was treated with aqueous phenol according to Kirby (3). The RNA fraction thus obtained was designated as 'pRNA'.

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After the extraction of pRNA, the phenol layer (the residue) was treated with excess volume of ethanol, and the protein precipitated by the addition of ethanol was then subjected to the procedure of Schmidt-Thannhauser to recover the residual RNA fraction ('rRNA'), which was not released by the method of Kirby. After the removal of the rRNA fraction, DNA was then extracted from the residue with sodium dodecyl sulphate by the method of Sibatani (4).

Each mononucleotide was separated by Dowex-1-formate ion-exchange chromatography. The specific activities of RNA's were calculated as the weighed mean of the specific activities of the mononucleotides; on the other hand, that of DNA was obtained directly.

Determination of RNA content was done by the phloroglucinol test (5), and the phosphorus content determined by the method of Gomori (6). The radioactivity of the samples was measured and their ultraviolet absorbancy determined spectrophotometrically, from which the phosphorus content was calculated in most cases.

The methods employed in the present experiment were essentially the same as those in the previous one (1), and the details will be published elsewhere.

Results

In the first experiment, 20 animals were inoculated and on the 10 th day after receiving an intraperitoneal injection of ^{32}P-orthophosphate, the animals were set free during 2 hours and then killed. The tumor cells were fractionated to obtain the nucleic acid fractions as described above.

Table 1. ^{32}P-orthophosphate incorporation into the nucleic acids of Ehrlich ascites tumor cells (10-day cells).

Fraction	Nucleotide	Total P (μg)	Molar ratio (AMP=10)	cpm/μg P	Total cpm	Specific activity
Total RNA	AMP	94.5	10.0	18.3	1730	15.7
	GMP	172	18.2	13.9	2390	
	UMP	101	10.7	17.8	1800	
	CMP	148	15.7	15.0	2220	
		(516.5)			(8140)	
rRNA	AMP	105	10.0	67.4	7080	65.2
	GMP	136	13.0	58.4	7940	
	UMP	108	10.3	71.0	7670	
	CMP	122	11.6	65.7	8020	
		(471)			(30710)	
pRNA	AMP	96.1	10.0	8.34	801	6.78
	GMP	189	19.7	5.78	1090	
	UMP	103	10.7	7.73	796	
	CMP	150	15.6	6.39	959	
		(538.1)			(3646)	
DNA	.....(μg P/ml=212, cpm/ml=1420).....					6.70

The specific activites of the total RNA, rRNA, pRNA and DNA fractions were 15.7, 65.2, 6.78 and 6.70, respectively. The detail of the results is presented in Table 1.

While the specific activity of rRNA is exceedingly higher than those of all others, the pRNA and DNA fractions give much lower values, and they are very close to each other, and that of the total RNA fraction is intermediate.

Concerning the specific activities of each alkali-produced mononucleotide from the three RNA fractions, it can be easily seen at first that guanylic acid usually exhibits the lowest activity and adenylic acid the highest with an exception in the case of the rRNA fraction (where the activity of uridylic acid is higher than that of adenylic acid) while the pyrimidine nucleotides, cytidylic and uridylic acids, generally give intermediate values. Contrary to the specific activity, on the other hand, the molar ratios of the nucleotides are roughly in inverse proportion, more or less uniform labeling being observed among the nucleotides within RNA, especially in the case of rRNA; a finding which agrees with the results of the other workers (for instance, 7). With respect to the nucleotide composition, high guanylic and cytidylic acid content are the characteristic pattern of the pRNA fraction, while in the rRNA fraction both of them are considerably lower.

In the next two experiments, in both of which 7-day cells were used as a starting material, the essentially same pattern was again observed as found in the first one. Table 2 shows the results obtained in one of the two experiments.

Table 2. ^{32}P-orthophosphate incorporation into the nucleic acids of Ehrlich ascites tumor cells (7-day cells).

Fraction	Nucleotide	Total P (μg)	Molar ratio (AMP=10)	cpm/μg P	Total cpm	Specific activity
Total RNA	AMP	117	10.0	39.4	4610	35.0
	GMP	211	17.9	30.3	6400	
	UMP	127	10.8	44.3	5630	
	CMP	177	15.1	30.6	5420	
		(632)			(22060)	
rRNA	AMP	83.4	10.0	194	1620	191
	GMP	101	12.1	180	1820	
	UMP	82.8	9.92	213	1760	
	CMP	93.6	11.2	181	1695	
		(360.8)			(6895)	
pRNA	AMP	128	10.0	21.8	2790	15.0
	GMP	253	19.8	11.9	3010	
	UMP	142	11.1	15.7	2230	
	CMP	198	15.4	13.9	2750	
		(721)			(10780)	
DNA	.....(μgP/ml=335, cpm/ml=5704).....					17.0

The specific activities of the total RNA, rRNA, pRNA and DNA fractions are 35.0, 191, 15.0 and 17.0, respectively. Also in this case, the specific activities of

pRNA and DNA are very close to each other, although not so close as in the first case.

On comparing the specific activities of the nucleotides, the situation is rather different from that of the first case. Uridylic acid, instead of adenylic acid, exhibits the highest activity in two of the three fractions, that is, in total RNA and rRNA, while in the pRNA fraction adenylic acid gives the highest value as found in the previous experiment. It seems, however, that the highest activity of uridylic acid is due to some incomplete removing of inorganic ^{32}P-phosphate when the nucleotides obtained by alkali-hydrolysis were separated by ion-exchange chromatographic technique employed throughout the experiments (8). Nevertheless, the low content of this nucleotide permits reliable calculation of the specific activity of the fraction as the weighed mean of the nucleotides.

The amount of ^{32}P-orthophosphate administered per animal is the same in both the first and second experiments, but the activities of the RNA fractions in the latter experiment are more than two times as high as in the former one, where old tumor cells (10-day cells) were used in place of young 7-day cells.

Using the phloroglucinol test, RNA composition was determined: 88 per cent of pRNA and 12 per cent of rRNA in the first experiment, 90 per cent of pRNA and 10 per cent of rRNA in the second one.

Discussion

On the basis of the results obtained with lymphatic cells of a rabbit appendix and either normal or regenerating rat liver, we have suggested that RNA in the cell can be fractionated by the treatment with aqueous phenol into two different species of RNA; one of them is the rather stable RNA which is now supposed to be involved, because of its specific activity significantly close to that of DNA, in the process of cell multiplication in some way, while the other species, turning over at an exceedingly higher rate, may be connected with general metabolism in the cell rather than directly with cell division.

Now the present results also confirmed our supposition and strongly supported the existence of the stable species of RNA in the tumor cells as well as in the tissues stated above. This species of RNA can be obtained by the treatment with aqueous phenol according to Kirby, while the other, which is highly active in incorporation of ^{32}P-orthophosphate, remains unextractable in the residue.

This observation suggests that the two RNA's are quite different not only in function but also in the mode of linkage to protein moiety; one is sensitive, and the other resistant, to the phenol treatment.

Another difference can be found between the nucleotide compositions of the two species of RNA. The pattern of high guanylic and cytidylic acid content is charac-

teristic of pRNA, and in rRNA, contrarily, adenylic and uridylic acid become relatively richer (see the Tables).

The high content of guanylic acid seems to have some interrelation with the low activity for uptake of radioactive phosphorus. In this respect, there are lines of evidence which supports this opinion. The large past of the pRNA fraction, obtained by the procedure described above, precipitates in M NaCl and its specific activity then becomes lower with increasing content of guanylic acid; RNA remaining soluble in the suppernatant shows a much higher activity, which was found to consist of a smaller amount of guaylic acid (1). This observation essentially parallels the results of Brown *et al.* (9), using Ehrlich ascites tumor. In the second place, the increase in the specific activities of the pRNA fraction was often accompanied by the decrease in guanylic acid content in regenerating rat liver (unpublished data). Finally, as easily recognized from the Tables, the specific activity of the pRNA fraction, which is rich in guanylic acid, is strikingly lower than that of the rRNA fraction, containing lesser amount of this nucleotide, the value of the latter being ten times higher than that of the former in the present experiments. Other evidence supporting this can be found in the previous reports (10).

It is, indeed, of the utmost interest that the pRNA fraction has a specific activity distinctly close to that of DNA in both proliferating and non-proliferating tissues, such as lymphatic cells of a rabbit appendix, normal and regenerating rat liver, and Ehrlich ascites tumor cells. From this fact, one can hardly escape the following conclusion: the rate of pRNA synthesis is just the same as that of DNA, and, moreover, pRNA duplication may take place, like DNA in many cells, at one particular phase of the cell division cycle.

If such is the case, it seems very likely that it may be pRNA, being synthesized with the same rate as that of cell division, which plays a fundamentally important role during the course of cell division, and then participates in the process of proliferation and differentiation of animal cells. Thus, it may not be unreasonable to expect that analysis of pRNA duplication mechanism may offer a clue to the elucidation of the cancerization process of the normal cells.

Only very little is known now about the natures of the two species of RNA, especially the possible interrelationship between the two, still remains a question wanting an answer. The possibility of rRNA, highly active species, being a precursor for the stable one (pRNA), is now being explored.

There is another point to be discussed here. Many works have already been done on the validity of the concept of conservation of RNA in animal and microbial cells to yield a variety of results. Some investigators have reported that RNA is well conserved during the experimental period (11). The others, however, have denied the conservatory nature of RNA (12). Such apparent discrepancy seems to be

principally due to the differences of biological materials used in their experiments. The workers, who recognized the conservation of RNA, had used rapidly growing tissues, such as mammalian cells in tissue cultuer, bacteria during long-phase growth, and neoplastic cells, while the other workers, who failed to find RNA to be conservative had employed as the materials the tissues in a somewhat resting state, such as adult mammalian cells.

According to our supposition, the amount of pRNA found in actively growing tissues must be more than in stationary ones, because this species of RNA is supposed to be involved in cell multiplication and, in fact, in the tumor cells studied in the present experiments, pRNA reaches 90 per cent of the total RNA (88–90 per cent). As compared with those obtained with the lymphatic cells of a rabbit appendix (70 per cent), and rat livers (85 per cent), one can easily admit that this value is significantly higher than the other two, and the RNA conservation recegnized in the rapidly growing cells may be ascribed primarily to this stable RNA (pRNA) which accounts for the greater part of the total RNA in the cell.

However, in addition to this stable RNA, there also exists in this system highly active RNA, which turns over at a considerably high rate. This finding is at variance with those of some workers (11), who have found complete conservation of RNA in their systems, but it should be emphasized that ^{32}P-orthophosphate was used as a tracer isotope in the present experiment, while in others other radioisotopes as well as orthophosphate were used. In this regard, the work of Révész *et al.* (13) is most interesting, who observed a 30 per cent loss of RNA purines for the first 48 hours, although the purine level was thereafter conserved at the constant 70 per cent level throughout the experimental period. However, it is difficult, at this time, to make an interpretation, and it is quite a fruitless effort until more is known about the nature and behaviour of the two species of RNA.

The last question that should be discussed here is the ditstribution of these two species of RNA among the subcellular fractions. Recently, it has often been observed by many workers (cf. 14), that after the administration of radioactive precursors, such as inorganic ^{32}P, the activity of nuclear RNA is always much greater than that of cytoplasmic RNA, differences among the cytoplasmic fractions being rather small. Furthermore, nuclear RNA (nRNA), according to Osawa and his coworkers (10), can be fractionated into two fractions differing in metabolic activity and extractability. There is little difference among the specific activities of $nRNA_1$ (extractable with neutral phosphate buffer), microsomal RNA and DNA, wherease that of $nRNA_2$ (residual nRNA) is much higher than those of the others. This observation obtained with rabit appendix, thymus and liver led them to the suggestion that these RNA's that is, $nRNA_1$ and microsomal RNA, as well as DNA, are being synthesized independently at nearly the same rate upon mitosis, and also suggested the possibility

of $nRNA_2$ being the precursor of microsomal RNA. With regard to the nucleotide composition of $nRNA_1$, microsomal RNA and $nRNA_2$, the same difference can be found between pRNA and rRNA. The content of guanylic and cytidylic acid is significantly higher in $nRNA_1$ and microsomal RNA than in $nRNA_2$ Thus, one cannot, at the present time, exclude the possibility that our pRNA corresponds to $nRNA_1$ and microsomal RNA, while rRNA to $nRNA_2$. The definite answer, however, may soon be obtained, since the distribution of RNA's among the subcellular fractions are now being explored in Sibatani's laboratory.

Summary

From Ehrlich ascites tumor cells, RNA was extracted according to the method of Kirby, which was designated as 'pRNA'. The residual RNA ('rRNA') was then extracted from the residue by the method of Schmidt-Thannhauser.

The pRNA fraction was very inactive in incorporation of ^{32}P-orthophosphate, and the specific activity of this RNA fraction was found to be very close to that of DNA; on the other hand, that of rRNA is strikingly higher.

The results obtained seemed to confirm our previous supposition and the difference of the two species of RNA was discussed.

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FORMATION OF NUCLEAR INCLUSION BODIES IN TISSUE CULTURE CELLS BY 4-NITROQUINOLINE N-OXIDE

(Plates VII and VIII)

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INTRODUCTION

Recent investigations have shown that many different types of carcinogens have in common, beside [I] carcinogenicity, [II] growth inhibiting action, [III] mutagenicity, and [IV] specific injurious action on cell nuclei and chromosomes.

4-Nitroquinoline N-oxide, a new type of powerful carcinogen, which was first demonstrated in 1957 by Nakahara and his associates (1), possesses also a potent carcinostatic action (2). Okabayashi (3) previously reported the mutagenicity of this compound on *Aspergillus*, while Mashima and Ikeda (4), more recently, observed an exceptionally strong mutagenic effect by applying this substance on certain biochemical mutants of *Streptomyces griseoflavus*. From these results biological actions [I], [II] and [III], enumerated above, may be said to be proved for 4-nitroquinoline N-oxide. There remains to be investigated the fourth action, the ability of producing specific damage of cell nucleus, which may also be possessed by this molecule.

In *in vivo* system, the results of interaction between the applied carcinogen or its metabolites and the cells may be expressed not only as various cell damages, but also as cell regeneration following local necrosis, and damages to the regenerating cells, while the change which is proximately responsible for carcinogenesis must be a mild one that does not inhibit cell division and simultaneously produce the essential inheritable change in the cell.

However, the damages of the cells depend not only upon the concentration of the drug, but also on the resistance and metabolic state of each cell. Therefore, if the cells to be acted upon can be made uniform, the grade of cell damage can be expressed only as the function of drug concentration. For this reason, in this study, we examined the grade of damages produced by 4-nitroquinoline N-oxide at various concentrations in tissue culture cells, which can be considered as more homogenous than *in vivo* system. In this way we attempted to elucidate the carcinogenic relations

of cellular responses, especially the intranuclear changes, and it was found that 4-nitroquinoline N-oxide produces a very characteristic change in cell nuclei, namely, the formation of nuclear inclusion bodies.

In this paper will be reported first our cytological and cytochemical findings on the nuclear inclusion body, to be followed by our experimental data which indicate that there is a parallel relation between the carcinogenicity of and the nuclear inclusion by 4-nitroquinoline N-oxide and its various derivatives.

Material and Methods

The tissue culture cells used in this study were the human liver cells (Chang) as a primary culture.

Chang's strain liver cells were obtained from the National Institute of Health, Tokyo, and maintained by us in our laboratory at Cancer Institute, by the procedure based on the principle of Nitta's method (5). The outlines of this procedure may be briefly described here: A healthy seven day old culture was processed with 0.01% sodium versenate solution to give a homogenous cell suspension. Following centrifugation of the suspension, the sedimented cells were resuspended in an aliquot of the fluid medium, which consisted of 8 parts of lactalbumin yeast extract saline and 2 parts of adult human serum. The lactalbumin-yeast extract saline was prepared by the addition of lactalbumin hydrolysate, yeast extract and dextrose to Earle's saline at the ratio of 0.5, 0.1, and 0.24 percent respectively.

For the replicate culture 1 ml of the cell suspension thus prepared was poured into a small square tube, in which a cover slip has been previously settled. The concentration of the inoculum was approximatately 10^5 cells per ml. After three days of incubation the old medium was removed and 0.9 ml of fresh medium was poured into to replace it.

The primary culture of the trypsinized mouse kidney cells was obtained from Dr. M. Endo (6) of the Institute for Infectious Diseases, Tokyo University, who cultured it in accordance with the modified Dulbecco's method (7). Namely, the mouse kidney cells, collected by the above method, which consisted of a large proportion of the proximal convoluted tubules and a small proportion of fibroblasts, were poured into a square culture tube by the similar technique to that in the case of liver cell culture. After every six days old medium was pipetted out and was replaced with 0.9 ml of fresh medium.

Beside 4-nitroquinoline N-oxide, the following substances were used in this study: 6-Chloro, 4-nitroquinoline N-oxide, 4-nitroquinaldine N-oxide, 4-nitro, 2-ethylquinoline N-oxide, 4-nitroquinoline, 6-nitroquinoline, 4-nitropyridine N-oxide, 4, 6-dinitroquinoline N-oxide, 9-nitroacridine N-oxide, 4-cysteinylquinoline N-oxide, and 4-bromoquinoline N-oxide.

All but the last of these compounds have been previously tested as to their carcinogenicity by Nakahara and his co-workers (8), who showed that the first four are strongly carcinogenic and the remaining six non-carcinogenic. Among the result of the carcinogenesis experiments with compound marked with have not been published. They are listed here according to the personal communication from Dr. Nakahara.

For comparison and in consideration of possiblly similar effects, some well known carcinogens of chemical groups unrelated to 4-nitroquinoline N-oxide were also tested on tissue culture cells. These carcinogens were: 20-methylcholanthrene, 3, 4-benzpyrene, nitrogen mustard, 3'-methyl-dimethylaminoazobenzene and beryllium salts.

In each test, 0.1 ml of the solution of the test substance was pipetted into a square culture tube, which contained 0.9 ml of fresh medium as above described. The solutions of the test substances were prepared with the aid of small amounts of alcohol or Tween 80, when necessary, but strict care was taken not to let their final concentrations exceed 1 percent.

After a period of incubation, the cover slip was taken out from the culture tube and was gently washed twice with physiological saline, fixed with ethanol-ether mixture (1 : 1), and stained with hematoxylin-eosin for microscopical examination.

Experimental Results

1. Formation of Nuclear Inclusion Bodies by 4-Nitroquinoline N-oxide

After repeated preliminary experiments it was determined that the final concentration of 10^{-5} M was the optimum dose of 4-nitroquinoline N-oxide for the most favorable cytological obervation. The incubation time was decided to be 24 hours in the case of Chang's liver cells and 9 hours for mouse kidney cells, also after preliminary trials at various intervals of incubation.

The common cytological changes observed in the both culture cells were the characteristic lesion in the nucleus, which consisted of eosinophilic precipitates surrounded by clear hallo, showing the typical picture of intranuclear inclusion bodies. They were independent from nucleoli. Other than these nuclear changes, occasional cytoplasmic degenerative signs were noticed: vacuolation, granulation, etc.

In Chang's liver cells, several of these precipitates with hallo were observed in one nucleus (Plate VII, figs. 3 and 4). These characteristic nuclear inclusions were seen only in the resting cells. Successively observed from about 6 hours of incubation, the appearance of small dotted eosinophilic precipitates was noticed in cell nuclei in about 5 percent of all the cells. This ratio increased gradually and reached about 50 percent at 24 incubation hours. At this time, however, the total cell number was decreased to one-half of that at the beginning. In the meantime the precipitates gradually increased in their volume and the hallo has also become increased in its clearness.

Nucleoli were surround with associated chromatin and remained unchanged throgh-

out this time. In both test and control cultures the average number of nucleoli per nucleus remained constant, that is, two or three.

In the mouse kidney cells it was usually observed that these precipitates gradually fused into one large mass and it often occupied the greater part of the nucleus. See Plate VIII, figs. 7 and 8.

The mitotic rate of the Chang liver cells was remarkably depressed already at 6 hours after the application of 4-nitroquinoline N-oxide, while the rate stayed at 3-4 percent in the control.

2. Cytochemical findings on the nuclear inclusion bodies.

The nature of the characteristic precipitates which were observed in the nuclei of tissue culture cells as described above, was analyzed by the application of several histochemical staining methods and the following results were obtained:

The precipitates (inclusion bodies) were negative to Feulgen as well as to PAs staining.

With pyronin the inclusions were stained positively, slightly more than the nucleoli. It was found that the stainability with pyronin remained unchanged after the treatment with ribonuclease, while this treatment completely digested out the nucleoli. Ribonuclease was applied according to the method of Brachet and Shave (9).

These data may indicate that the substance composing the nuclear inclusion may be depolymerized DNA or basic protein.

Parallel Relation between the Inclusion Body Formation and Carcinogenicity of 4-Nitroquinoline N-oxide Derivatives and Allied Substances

The question of whether or not the intranuclear changes which were produced in tissue culture cells by 4-nitroquinoline N-oxide can be induced also by the action of its derivatives was studied in this experiment.

In most cases the effects were tested on both Chang's liver cell and mouse kidney cell, incubation time in the case of the former being 24 hours, and for the latter 9 hours. 9-Nitroacridine N-oxide, 4-bromoquinoline N-oxide, and 4-cysteinylquinoline N-oxide were tested only upon Chang's liver cells. The final concentrations of the test substances were chosen after many preliminary experiments as optimum for the purpose of the tests.

The results of this experiment are shown in Table 1, in which are listed the substances and the final concentrations at which they were tested, nothing their ability to produce the characteristic intranuclear inclusions, and comparing these results with carcinogenicity based on the data of Nakahara and co-workers. A parallel relation between the nuclear inclusion formation and carcinogenic potency is striking.

The nature of the characteristic nuclear inclusions produced by 4-nitroquinoline

Table 1. Ability of forming nuclear inclusion of 4-nitroquinoline N-oxide derivatives and other related compounds in comparison with their carcinogenicity.

Compounds	Final concent. used	Inclusion formation	Carcino-genicity
4-Nitroquinoline N-oxide	10^{-5}M	+	+
6-Chloro, 4-nitroquinoline N-oxide	10^{-5}M	+	+
4-Nitroquinaldine N-oxide	10^{-5}M	+	+
2-Ethyl, 4-nitroquinoline N-oxide	10^{-5}M	+	+
4-Nitroquinoline	10^{-5}, 10^{-4}, 2.5×10^{-4}M	−	−
4-Nitropyridine N-oxide	10^{-5}, 10^{-4}, 5×10^{-4}M	−	−
6-Nitroquinoline	10^{-5}, 10^{-4}, 10^{-3}M	−	−
4, 6-Dinitroquinoline N-oxide	10^{-5}M	−	−
9-Nitroacridine N-oxide	10^{-5}, 10^{-4}M	−	−
4-Bromoquinoline N-oxide	10^{-5}, 10^{-4}, 10^{-3}M	−	−
4-Cysteinylquinoline N-oxide	10^{-5}, 10^{-4}, 10^{-3}M	−	−

N-oxide derivatives was entirely identical with those by 4-nitroquinoline N-oxide itself.

As to those substances which did not produce the nuclear inclusions, fnrther experiments were carried out using higher concentrations up to the point just below that which caused the detachment from the cover slip of monolayer of culture cells. In no case was it possible to observe the formation of inclusion bodies.

Cytological effect of known carcinogens chemically unrelated to 4-Nitroquinoline N-oxide

It now becomes important to investigate whether carcinogenic substances, other than those of the 4-nitroquinoline N-oxide group, can be shown to have the ability to produce nuclear inclusions in tissue culture cells. For this purpose we selected 20-methylcholanthrene, 3, 4-benzpyrene, nitrogen mustard, 3'-methyl, dimethylaminoazobenzene, beryllium nitrate, beryllium sulfate and beryllium chloride.

The two hydrocarbons, first listed, were tested at 10^{-8}M concentration on both Chang's liver cell and mouse kidney cell. Nitrogen mustard was used at 5×10^{-5}M, on Chang's liver cell only. The aminoazo dye was tested on Chang's liver cell at 10^{-5} and 10^{-4}M concentrations. Beryllium salts were tested also on Chang's liver cell at the concentrations of 10^{-5}, 10^{-4}, and 10^{-3}M.

The formation of the nuclear inclusions was not observed in any of these experiments, in spite of the fact that the carcinogens were tested up to the highest usable concentrations. The more difficultly soluble substance, the azo dye, was applied as homogenized suspensions, however.

Discussion

Our expectation that 4-nitroquinoline N-oxide, preeminent as carcinogen, possessing

also anti-cancer action as well as mutagenicity, may produce some specific damage of cell nucleus has been fulfilled. It has now been shown that this substance produces at a certain range of concentrations characteristic intranuclear inclusion bodies in in tissue culture cells.

Generally, the inclusion bodies are divided morphologically into cytoplasmic and nuclear types. They are usually observed in virus infections, and particularly the nuclear inclusions have been noted in the case of the DNA virus infections. However, the formation of intranuclear inclusions have been reported under various conditions other than virus infections, and those in hepatic parenchymal cells have been observed under a wide variety of conditions. Thus, Wilson and Leduc (10) reported nulear inclusions in the liver of mice maintained on diet containing 50 percent bentonite, and the increased frequency of inclusions when methionine was added to the bentonite diet (11): Hamperl (12), Buchman (13) and Lambers (14) in mouse liver in acute and chronic colchicin intoxication: Zollinger (15, 16) in kidney epithelium in lead poisoning and after Roentgen irradiation: Rather (17) and Kleinenfeld et al. (18), in mouse liver in thioacetamide intoxication. More interesting perhaps are the findings that nuclear inclusions occur also in cancer cells (Zollinger (16)), as also reported by Leduc and Wilson (19) for a transplantable hepatoma. In addition. Hirokawa (20) found nuclear inclusions in granuloma produced in guinea pig by intraperitoneal injections of beryllium oxide, and Shelton (21) noted similar inclusions in the course of hepatic carcinogenesis by aminoazo dye feeding.

The fact that the formation of nuclear inclusions does take place, not only as the specific expression of virus infection, but also from various other causes, permits the inference that biochemical processes, which are not specific to virus infection, may bring about the formation of intranuclear inclusions.

The nuclear inclusion produced in tissue culture cells under the influence of 4-nitroquinoline N-oxide is a corpuscular substance surrounded by a hallo, which develops either singly or in number in the resting cells, totally without relation to nucleoli. Morphologically, it resembles most closely the nuclear inclusion in measles infection. Cytochemically, the presently available date indicate that it may be depolymerized DNA or basic protein. Cytological details, especially its relation to nuclear membrane and the early morphology of its formation are now under investigation in this laboratory, but there seems to be no doubt that its formation is an expression of a certain definite biochemical process.

It is true that 20-methylcholanthrene, 3, 4-benzpyrene, nitrogen mustard, aminoazo dye and beryllium salts, in spite of their carcinogenicity, do not produce nuclear inclusion in tissue culture cells, under the condition in which the inclusion bodies are so readily formed by 4-nitroquinoline N-oxide. In our opinion, this discrepancy does not repudiate the significance of the inclusion body formation by 4-nitroquinoline N-

oxide in its carcinogenic activity.

It is to be expected that different types of carcinogenic substances have their own proper metabolic pathways *in vivo*, and it is conceivable that each of them, in one of its metabolic steps, may produce its specific damage in cell nucleus. These specific damages may be morphologically undetectable in some cases, or may be clearly visible as chromosome rupture or bridge formation as in the case of nitrogen mustard. On the other hand, the failure to produce nuclear changes in tissue culture cells by aminoazo dye or beryllium, already reported to form the inclusions *in vivo*, may mean that the formation of the inclusions by these agents takes place under a complex mechanism and requires a longer time than attainable in the tissue culture condition. The constant and rapid formation of the nuclear inclusion in tissue culture cells by 4-nitroquinoline N-oxide probably depends on the relative homogeneity of the cell population, as well as on the extreme simplicity and directness of the mode of action of this substance.

It may be also noted, in passing, that we have so far failed to demonstrate the formation of the nuclear inclusions in the mouse skin so treated as to produce skin cancer with 4-nitroquinoline N-oxide. This may be due to the different biological responses of skin epithelial cells as conditioned by their intrinsic character and metabolic activity, resulting in too small a number of cells receiving the optimum, moderate damage for the inclusion formation to be recognizable by microscopical examination.

Insofar as concerns 4-nitroquinoline N-oxide and related substances, there is no doubt that their carcinogenicity and potency to form nuclear inclusions in tissue culture cells are completely paralleled, suggesting the close relation between the two properties. The failure of non-carcinogenic derivatives to form the inclusion is not a matter of the diffence in effective concentrations, since these inactive derivatives do not produce the inclusion even at the highest usable concentration, beyond which their toxicity brings about the detachment of culture monolayers.

Just how the nuclear inclusion formation is related to carcinogenic mechanism is a question which cannot be answered at the present stage of our study. One way of approaching this question may be to trace the fate of the cells containing the inclusion. In this connection, the possibility should not be ignored that the inclusion formation, just as chromosome breakages produced by mustards, may be too drastic a change to determine the cancerous transformation of normal cells. It may be recalled that 4-nitroquinoline N-oxide at the optimum inclusion-forming concentration (10^{-5}) induces considerable inhibition of cell division. It is therefore possible that the inclusion formation may be a stage leading to cell death, and, in itself, unrelated to carcinogenic process.

The important problem here is that of the *milieu* in which the nuclear inclusion

comes into existence. It is readily conceivable that among the apparently intact cells, co-existing with those containing the inclusion bodies, there may be cells which have undergone the essential inheritable change. In this sense, even if the inclusion formation itself may eventually be shown to be a step toward cell death, its existence may be a significant indication of the existence in the same *milieu* of other cells which are being transformed into cancer cells.

Summary

By the application of a new type of powerful carcinogens, 4-nitroquinoline N-oxide and some of its derivatives, on tissue culture cells, characteristic intranuclear inclusion bodies were produced in the resting cells. From histochemical point of view, these nuclear inclusions may be considered to be depolymerized DNA or basic protein in nature.

The complete coincidence between the carcinogenicity and the ability of the nuclear inclusion formation was observed among 4-nitroquinoline N-oxide and its derivatives and related compounds, but carcinogens of unrelated chemical groups failed to give rise to similar nuclear inclusions.

We wish to express our hearty thanks to Dr. Waro Nakahara for his interest and encouragement throughout this study.

We are indebted to Dr. M. Endo of Institute for Infectious Deseases of Tokyo University and to Dr. M. Yamada and Dr. K. Takano of National Institut of Health, who have supplied us the cultured cells.

Our thanks are also due to Miss T. Fujiwara for her technical assistance.

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EXPLANATION OF FIGURES (Plates VII and VIII)

Figs. 1–4. Chang's strain liver cells, 24 hour culture.

Fig. 1. Untreated control.

Fig. 2. Treated with 10^{-5}M 4-nitroquinoline N-oxide.

Fig. 3. Same as Fig 2, higher magnification, showing intranuclear inclusion bodies.

Fig. 4. Same as above, still higher magnification, showing structural details of inclusion bodies.

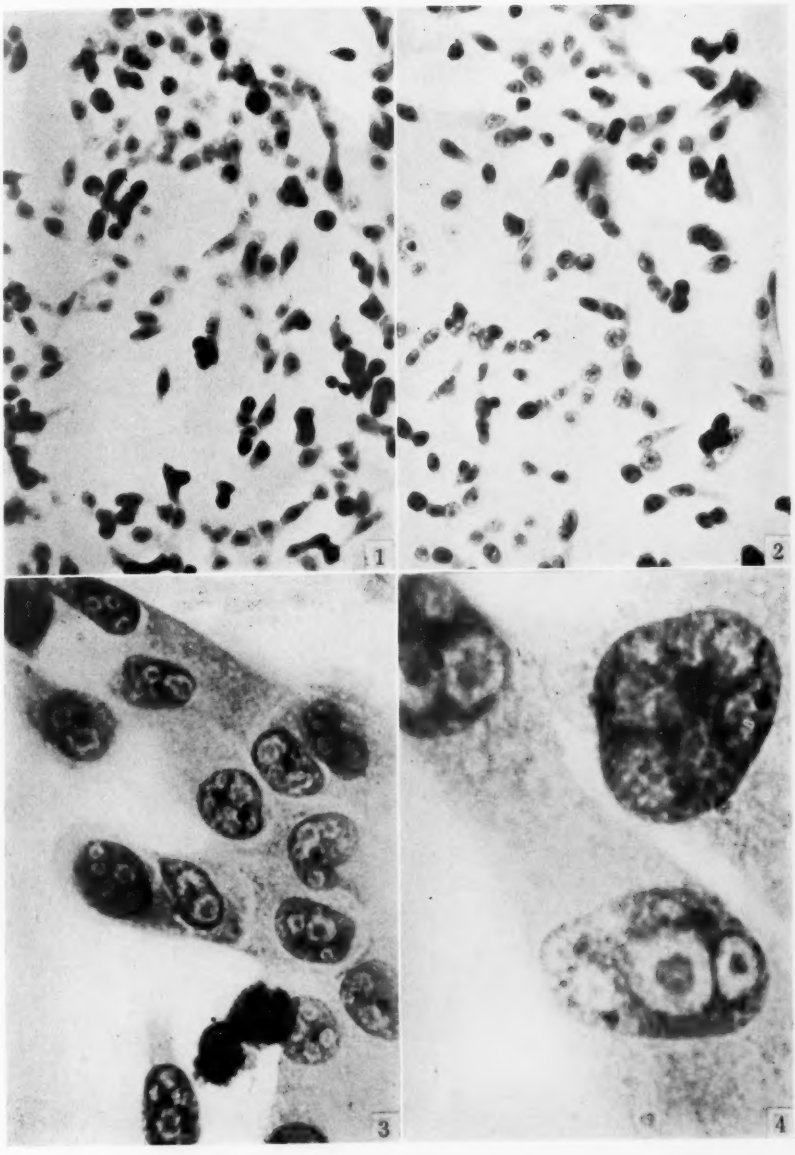
Figs. 5–8. Mouse kidney cells, 9 hour culture.

Fig. 5. Untreated control.

Fig. 6. Treated with 10^{-5}M 4-nitroquinoline N-oxide.

Fig. 7. Same as Fig. 6, higher magnification, showing nuclear inclusions.

Fig. 8. Same as above, still higher magnification, showing three nuclear inclusion bodies in a vacuolated cell.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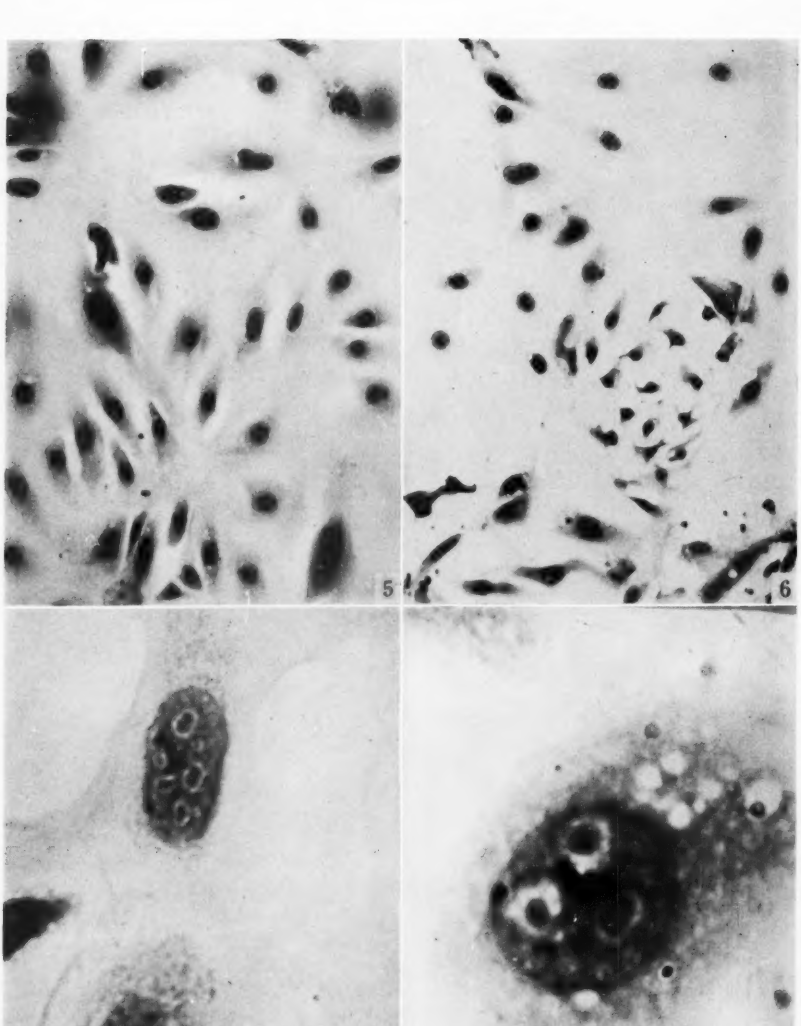
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[GANN, 50, 219~226; September, 1959]

HISTOLOGICAL AND HISTOCHEMICAL STUDY OF THE EARLY LESIONS OF MOUSE SKIN AFTER A SINGLE APPLICATION OF 4-NITROQUINOLINE N-OXIDE

(Plates IX-XII)

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INTRODUCTION

In 1957, 4-nitroquinoline N-oxide was demonstrated to be a new type of chemical carcinogen by Nakahara[1] and his colleagues. This substance is characterized not only by its high carcinogenic potency but also by the simplicity of its chemical reaction[2] *in vitro*. The reaction, as reported by Okabayashi[3] (1953) and confirmed by Endo[4] (1958), is that of substitution with SH-group, which takes place rapidly within the physiological pH range without enzymic intervention. It has been also known[3,4] that under the similar condition 4-nitroquinoline N-oxide does not react with R-COOH, R-NH_2, ATP, RNA or DNA. According to Endo[4] a definite correlation exists between the reactivity with SH-group *in vitro* and the carcinogenicity[9] of various quinoline derivatives.

All these facts suggest that such a reaction may be assumed to take place also *in vivo* as the initial step of its biological actions. However, this assumption must be confirmed by direct experimental evidence. The present study is attempted in order to find if the 4-nitroquinoline-N-oxide actually react with SH-group of the epidermal cells *in vivo*.

In this paper will be reported, (1) some histological changes of mouse skin during the first 48 hours after a single application of 4-nitroquinoline N-oxide and (2) the correlation between the occurrence of such morphological lesions and changes of intraepidermal SH-content. The skin reaction to 4-nitroquinoline N-oxide will be compared with those to a variety of other quinoline derivatives.

MATERIALS AND METHODS

270 male mice of DD-strain were used, all weighing around 18g, and maintained on the standard diet in our laboratory. They were divided into three groups according to the experimental designs (Table 1).

The solution to be tested was applied with a capillary pipett to the interscapular region of mouse skin. In Group III, four kinds of solution in different concentrations

Table 1. Experimental groups.

Group	Research-objective	Painted material	Number of mice
I	Histological and histochemical observation ot the skin 1, 2, 3, 6, 12, 24 and 48 hours after a single painting.	0.25% 4-nitroquinoline N-oxide in acetone.	25
		0.25% 4-nitroquinoline N-oxide in benzene.	40
		0.25% quinoline N-oxide in acetone.	25
		Acetone.	25
		Benzene.	20
II	Chemical analysis of protein-bound thiol-group in the epdermis, 1, 3, 6, 12, and 48 hours after the painting.	0.25% 4-nitroquinoline N-oxide in acetone.	40
		Acetone only.	40
		None (control).	20
III	Histological comparison of the skin lesions by various quinoline derivatives in a serial concentration (in acetone), 10^{-1}, 2×10^{-2}, 4×10^{-3} and 8×10^{-4}M.	4-Nitroquinoline N-oxide.	5
		4-Nitroquinaldine N-oxide.	5
		4-Nitroquinoline N-oxide 6-sulfonic acid.	5
		4-Nitroquinoline N-oxide 2-propionic acid.	5
		4-Nitroquinoline.	5
		6-Nitroquinoline.	5
		Quinoline N-oxide	5

were applied to four separated regions of the dorsal skin of each animal. The hair had been pulled off 24 hours before the treatment.

Histological procedures: Excised sample of the skin was placed on sheat of filter paper and fixed in ethanolformaline (1 : 1) for 24 hours. Hematoxyline-eosine staining of paraffine sections were made according to our routine method.

Histochemical procedures: Barnett-Seligman's DDD method[5,6] was adopted with one modification: 25mg of DDD (22'-dihydroxy, 66'-dinaphthyl disulfide) was dissolved in the solvent, prepared by mixing 25 ml of absolute ethanol with 25 ml of 0.1M veronal buffer (pH 8.9), instead of 15 ml of absolute ethanol and 35 ml of 0.1M veronal buffer (pH 8.5) in their original description. Other procedures were the same as the original method.

Procedures for quantitative analysis of protein-bound SH-group:[7,8] Preparation of sample: The excised skin flap was placed immediately on a paraffine developer at 45°C for 20 seconds. The epidermis was separated with small forceps from the

subjacent structures. The separated epidermis was washed 5 times with 5cc of ether and dried. After drying, the material was pulverized in an agate mill.

One or two mg of pulverized sample was flocculated in 0.5 ml of 10% solution of duponol in water and boiled for 10 min. 5 ml of Bennet's reagent was added to it. After mechanical shaking for 20 min. at 250 osscillation per min, the solution was centrifuged at 2000 rpm for 15 min. 3 ml of the supernatant was put in a test tube and 1.5 ml of conc. HCl was added. After enough shaking, the tube was left standing for 15 min. Absorption was measured by Beckmann's photometer (Type-DU) at 510 milli-micra of wave-length.

Results

Gross findings; In the 4-nitroquinoline N-oxide group, slight edematous swelling was noticed in the treated areas of the skin within the first 6 hours, and the epidermal surface became seborrhoic. At 12 hours, the edematous changes reached the acme. Afterwards, the epidermis appeared atrophic and scaley exfoliation occurred. Occasional small erosions were seen in some cases. At 48 hours, most parts of the epidermis were thickend and the surface was covered with a mixture of cornified and necrotic materials. In the dermis conspicuous edema, hyperemia and scattered petechiae were seen at the first 1 hour, which persisted throngh the whole course.

In the control groups, slight edema and seborrhoic state of the surface were observed in the earliest stage. After 24 hours, the epidermis became slightly thickened and pityriatic. Intradermal inflammatory changes were observed at 1 hour. However, hemorrhagic lesions were observed only exceptionally.

A. Histological findings

1. *4-Nitroquinoline N-oxide* (Group-I, 1): Within 2 hours after the painting, slight cytoplasmic and nuclear vacuolation was observed in some parts of the basal layer of the epidermis. After 3 hours intra- and intercellular edema was found in the epidermis, which became rapidly severe and developed to intraepidermal vesicle or epidermodermal cleavage. Such lesions reached maximum around 12 hours after the treatment in association with the extensive appearance of degenerating or necrotizing processes of cells there. At 24 hours, the necrotic lesions were most conspicuous, which occupied most of the superficial layer of the treated area and extended to some part of hair-follicles. In this state, regenerating process or cell-proliferation appeared especially at the upper portions of hair-follicles and in the epidermis around the necrotic area. At 48 hours, most parts of the epidermis were thickened and the surface was covered by necrotic and cornified materials. A few small ulcerous lesions might be left.

Inflammatory changes in the dermis—vascular dilatation, edema or cell infiltration—were observed very soon after the painting and still persisted at 48 hours. The

most of the sebaceous glands became atrophic after the treatment and some of them were necrotized where the hair-follicles were severely attacked.

2. *4-Nitroquinoline N-oxide in benzene* (Group-I, 2): Little qualitative difference in the skin lesions was observed when the solvent was benzene instead of acetone. However, the lesions appeared more rapidly and became severer. Vesiculations were noticed 1 to 3 hours after the treatment.

3. *Acetone and benzene* (Group-I, 4 and 5): Vesication was not found in either cases of the two kinds of solvent only. Slight spongiotic changes were observed 12 hours after benzene painting. Occasional slight necrotizing effects were also noticed in benzene case. In both groups, parakeratosis of the treated epidermis was observed. Atrophy of the sebaceous glands and intradermal inflammatory reaction were noticed in both cases, which were more conspicuous in the benzene-group.

4. *Quinoline N-oxide in acetone* (Group-I, 3): Skin lesions were comparable to those with acetone only. The reactions appeared even less conspicuous although slight acanthosis followed at 48 hours.

B. Histochemical findings

1. *Normal control:* Diffuse and moderate coloration was found in the basal and Malpighian layers of the epidermis, upper portion of the hair-follicles and hair-bulbs. Much intense coloration was seen in the outer- and inner-sheaths and prekeratogenous zones of the hairs. In the cornified layer of the epidermis and fully keratinized part of the hairs, no reaction was observed. In the dermis and subcutaneous tissue, slight coloration was noticed in some connective tissue cells and moderate one occurred clearly along the striations of the muscle cells.

2. *4-Nitroquinoline N-oxide group:* In the early stage of the lesions, only very slight dropping of staining was noticed. Marked decrease of coloration occurred in the vesicated parts of the epidermis. It was most conspicuous in the superficial necrotized layer at 24 hours. At 48 hours, slightly intensified coloration became observable in some parts of the superficial layer of the acanthotic epidermis.

3. *Quinoline N-oxide group and acetone group:* During the first 12 hours, noticeable changes in the stain reaction did not occur. After 12 hours, moderate intensified coloration appeared in the superficial layer of the epidermis assocociated with parakeratosis.

C. Chemical finding

The normal value of SH-group in the mouse epidermis was calculated to be around 0.06 micro-mol / mg dry weight by referring to the standard calibration, which was obtained with 0.02 to 0.2 micro-molar solution of dl-cystein in water. SH-content of epidermis in the experimental groups were presented in Chart 1 as the differience of their value from the normal.

In the 4-nitroquinoline N-oxide group, evident decrease of SH-content was already

noticed at 1 hour (about -25%), which became most obvious at 12 hours (about -40%) and still noticed at 48 hours. In the acetone group, slight decrease (about -10%) was noticed within 12 hours, which was almost recovered at 24 to 48 hours.

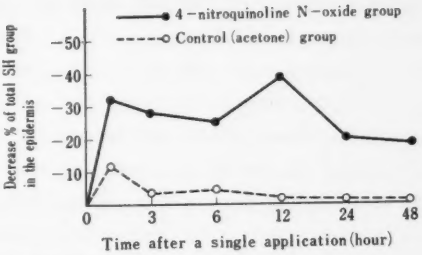

Chart 1. Changes of SH-content in the epidermis.

D. The comparison of vesication-potency of various quinoline derivatives (Group-III):

Table 2 shows the ratio of the numbers of animal with vesication to the total numbers of the mice used in each group.

Table 2. Occurrence-rate of vesicle in each group
(Number of animal with vesicle / total number)

Material \ Concentration (M) in acetone	8×10^{-4}	4×10^{-3}	2×10^{-2}	10^{-1}
4-Nitroquinoline N-xide	1/5	2/5	4/5	5/5
4-Nitroquinaldine N-oxide	1/5	2/5	3/5	5/5
4-Nitroquinoline N-oxide 6-sulfonic acid	0/5	2/5	2/5	3/5
4-Nitroquinoline N-oxide 2-propionic acid	0/5	1/5	2/5	3/5
4-Nitroquinoline	0/5	1/5	0/5	0/5
6-Nitroquinoline	0/5	0/5	0/5	1/5
Quinoline N-oxide	0/5	0/5	1/5	0/5

The vesication-potency of tested quinoline derivatives may be arranged in the following order:

4-Nitroquinoline N-oxide, 4-Nitroquinaldine N-oxide > 4-nitroquinoline-N-oxide 6-sulfonic acid, 4-nitroquinoline N-oxide 2-propionic acid ≫ 4-nitroquinoline, 6-nitroquinoline, Quinoline N-oxide, Acetone only.

Apparent parallelism exists between their vesicant potency and reactivity with SH-group *in vitro*.[4]

Discussion

In this study it was clearly observed that 4-nitroquinoline N-oxide caused certain histologically characterized changes to the mouse skin within the first 24 hours after its single application. They were characterized by the occurrence of intraepidermal vesicles and/or junctional cleavages associated with degenerative, sometimes necrotizing, changes of the epidermal cells and severe exudative reaction in the dermis. Although some of the epidermal and dermal changes were also seen in the control

groups (treated with solvent only or quinoline N-oxide), tne epidermal vesiculation and the junctional cleavage could be regarded as specific reaction to 4-nitroquinoline N-oxide among the chemicals tried in this series.

By the histochemical method, the occurrence of the skin lesions was found to be closely correlated with the decrease of intraepidermal SH-content. This fact was confirmed further by the chemical analysis of the treated epidermis which showed 25-40% drop in the 4-nitroquinoline N-oxide group.

The lesions are quite comparable with the description of Dixon and Needham[10] (1946) and Cullumbine[11] (1947) in their respective study of vesicant action to the skin.

The role of SH-groups in the mechanism of vesicant action to the skin has been frequently discussed. In 1946, Dixon and Needham[10] reported the fact that among various SH-blocking agents a correlation existed between the hexokinase inhibiting action and vesicant-potency. Peters[12] (1947) also reported that the initial action of vesicants was the blocking of SH-groups at the cell surface. He also emphasized a clear-cut inhibiting effect of the vesicants on pyruvate-oxidase activity. Marcus[13] (1950), Flesh[14] (1950), Danielli[15] (1953) and Rothman[16] (1954) also supported the view correlating the vesicating action with SH-blocking effect of the chemical.

The above data strongly suggest that 4-nitroquinoline N-oxide actually undergoes a reaction with some intraepidermal SH-groups *in vivo*. This possibility was further confirmed by the experimental fact in Group III, that among various quinoline derivatives the potencies causing such skin lesions were definitely conicident with their reactivity to SH-groups *in vitro* (Table II). There was, however, one exception: 4-nitroquinoline N-oxide 6-sulfonic acid. Contrary to the fact that the reactivity of this substance with SH-group *in vitro* was known to be higher[17] than 4-nitroquinoline N-oxide, its vesicating action was much lower that that of 4-nitroquinoline N-oxide. This phenomenon may be explained by the lesser permeability[18] of this substance through the cell membrane due to the presence of hydrophilic radical at position 6.

Recently di Paolo[19] (1958) reported that painting of carcinogens caused an early fall in SH-content of the epidermis followed by a transient increase before it returned to normal level. Such a diphasic changes in the epidermal SH-content was not observed in the case of 4-nitroquinoline N-oxide. In the present study, in spite of localized intensification of the histochemical reaction at the portion with dyskeratosis, overall SH-content of the epidermis showed a monophasic decrease.

Summary

1. Correlative study of histological, histochemical and biochemical changes occuring in the mouse skin within 48 hours after a single painting of 4-nitroquinoline N-oxide and other quinoline derivatives is reported.

2. The early histological skin lesions caused by 4-nitroquinoline N-oxide were consistent with the vesicant actions.

3. The occurrence of these skin lesions were associated with the decrease of intraepidermal SH-content in the treated areas.

4. Among a variety of quinoline derivatives, the potencies causing these skin lesions were definitely correlated with their reactivity with SH-group *in vitro*.

5. These data indicate that 4-nitroquinoline N-oxide reacts with SH-group also *in vivo* as the first step of its biological action.

The author wishes to express his thanks to Dr. Kunio Oota, under whose guidance the present work was done. Thanks are also due to Dr. Waro Nakahara, to Dr. Hideya Endo and to Dr. Shozo Takayama for their helpful suggestions.

The samples of 4-nitroquinoline N-oxide, quinoline N-oxide and 4-nitroquinoline were prepared for me by Prof. Eisaku Hayashi (Shizuoka Pharmaceutical College), to whom my thanks are due.

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Expplanation of figures (Plates IX—XII)

Fig. 1. Skin of an untreated control mouse. H.E. 100×.

Fig. 2. 3 hours after a single application of 4-nitroquinoline N-oxide. Moderate intercellular edema of the epidermis. H.E. 40×.

Fig. 3. 6 hours after a single application of 4-nitroquinoline N-oxide. Conspicuous intraepidermal vesiculation. H.E. 100×.

Fig. 4. 24 hours after a single application of 4-nitroquinoline N-oxide. Demarcation of necrotic epidermal tissue from subjacent structure. H.E. 100×.

Fig. 5. Regeneration process at the basal layer of the epidermis, 24 hours after a single application of 4-nitroquinoline N oxide. H.E. 200×.

Fig. 6. 48 hours after a single application of 4-nitroquinoline N-oxide. Acanthotic epidermis of the skin. The surface was covered by necrotic and cornified materials. H.E. 100×.

Fig. 7. 12 hours after a single application of acetone. Slight thickening of the epidermis and atrophy of the sebaceous glands. H.E. 200×.

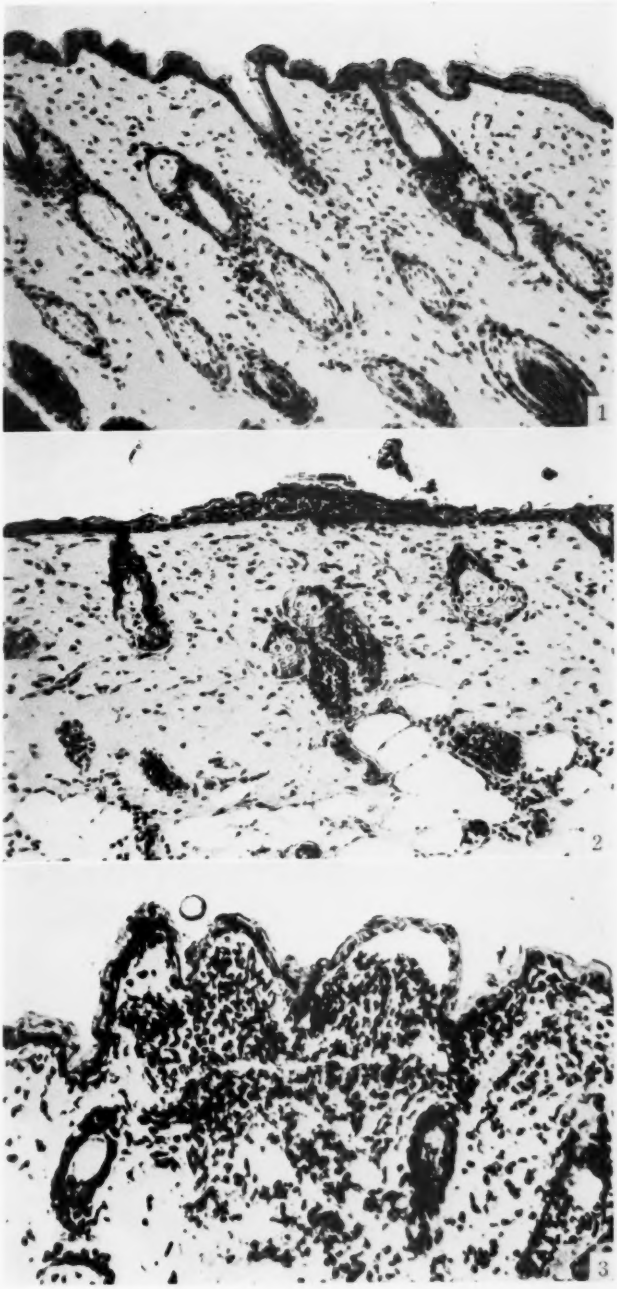
Fig. 8. 48 hours after a single application of quinoline N-oxide. Slightly acanthotic and parakeratotic epidermis. H.E. 100×.

Fig. 9. Skin of an untreated control. DDD. 200×.

Fig. 10. 6 hours after a single application of 4-nitroquinoline N-oxide. Decrease of coloration in the vesicated epidermis. DDD. 200×.

Fig. 11. 12 hours after a single application of 4-nitroquinoline N-oxide. DDD. 200×.

Fig. 12. Acanthotic epidermis 48 hours after a single application of 4-nitroquinoline N-oxide. Intense coloration at the dyskeratotic portion. DDD. 200×.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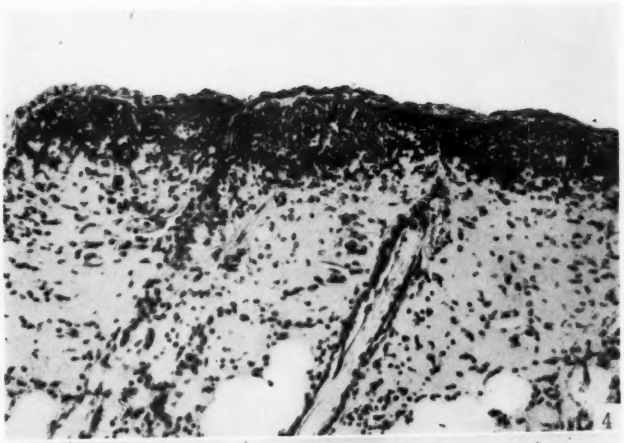
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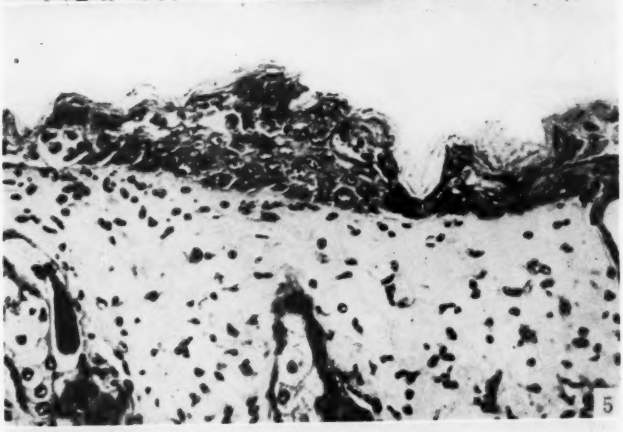
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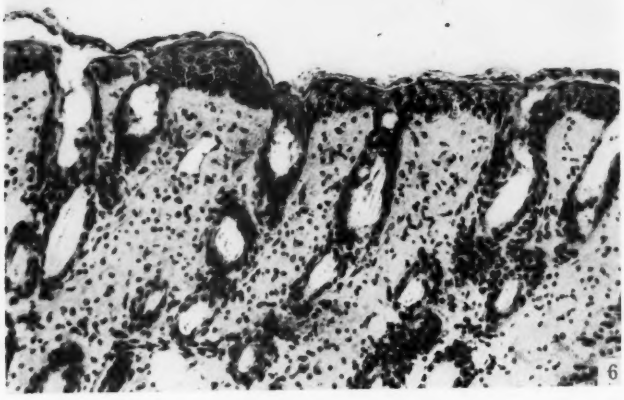
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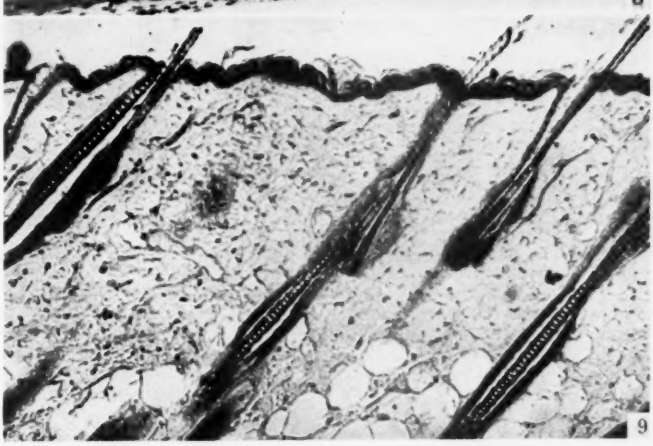
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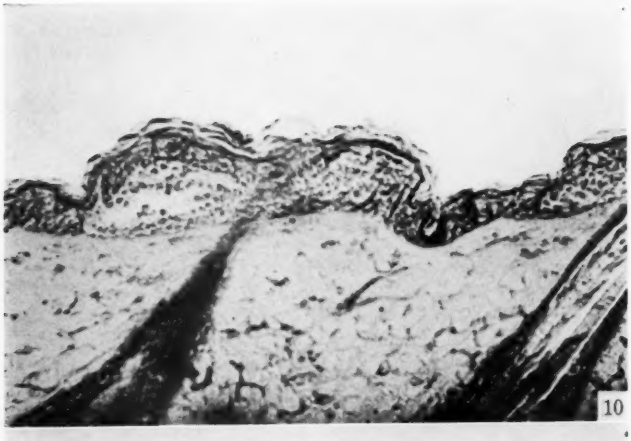
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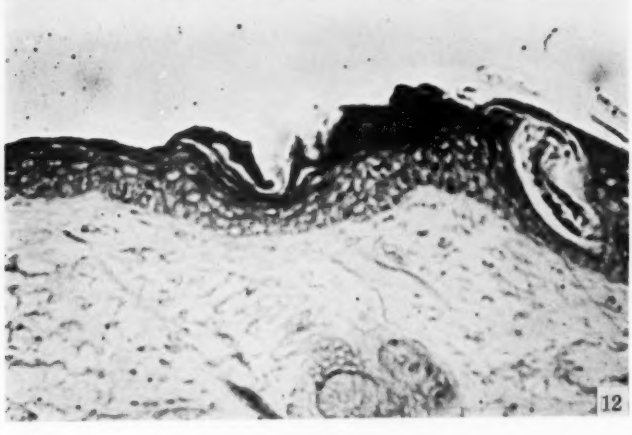
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[GANN, 59, 227~231 ; September 1959]

TUMOR FORMATION BY IMPLANTATION OF THE ESTABLISHED HUMAN AMNION TISSUE CULTURE CELL LINE "H" IN X-IRRADIATED AND CORTISONE TREATED RATS

(Plates XIII and XIV)

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The expedient of Toolan's method in 1954 (1, 2) has provided the ample scope for the experimental oncology through the introduction of the successful maintenance and passages of human tumors in x-irradiated and cortison-treated rats. To be specific, the numerous meritorious works on the production of tumors by transfer of established tissue culture cells into the conditioned hosts were carefully reviewed by Coriell *et al.* (3, 4) in reference to the alteration of cells from normal origin during the cultivation *in vitro* with the criteria for determining malignancy.

The impetus for the desirability to obtain human tumors at ease in the laboratory scale has led us to the examination of the human amniotic cells "H" strain which was established by us in 1957. The tumour produced by implantation of the cultured cell strain was initially documented by Fogh *et al.* (5). The surface properties of this tumor was duly compared with original cell lines with the assistance of the immuno-cytopathgenic technique of Mountain (6).

Matarials and Method

Cell culture: Growth medium consisted of 20 parts of human serum in 80 parts of LY medium (7) with the addition of 100 units penicillin and 50 gamma streptomycin per ml. Passage of cell cultures were made through 0.25% trypsin Difco in Hank's BSS. Hela cells were originally obtained from the National Institute of Health of Japan. Human amnion cell line "H" was established by the similar procedure published by Fogh *et al.* (7). Tumor cell cultures in this experiment were made by mincing the mass followed by trypsinization and subsequent introduction into growth medium milieu. Control primate amnion cells from normal fresh membrane were made from 1 cm^2 pieces by repeated trypsinization with stirrer. Viability of cells were tested by neutral red.

Conditioning of rats: Male albino rats weighing 40–45g were irradiated with 200r of hard X ray 4–6 days before implantation. (200 KUP 25 ma through 0.5 Cu and 0.5 Al filter, 80.6 r/min. at a focus distance of 50 cm. Tooshiba KXC 18). 3 mg of

cortisone was given at the day of implantation followed by three shots of same dose every other day.

Cytopathogenic antisera: Both anticellular serum for H cells and HeLa cells were prepared in rabbits by the method of Mountain (6), stored at 4°C for 2 months before employment.

Experimental Results

1) The incidence of tumor production with H and HeLa cells.

The 50 th passage cells of H strain in the amount of 0.1 ml (4×10^7 cells per ml.) and HeLa cells (6×10^7 cells per ml.) were independently introduced subcutaneously into the inguinal region of pretreated rats followed by post-cortisone treatment. The comparison is shown in Table 1. The positivity was confirmel by histological findings

Table 1. Result of the Implantation of H- and HeLa-Cell in the Irradiated and Cortisone-treated Rats.

H-Cell implanted rats			HeLa-Cell implanted rats		
Exp. No.	Tumor formation	Size	Exp. No.	Tumor formation	Size
1	−		1§	+	0.7×1.0×0.5
2*	+	1,5×0.7×0.7cm	2	−	
3	+	0.5×0.5×0.5	3	−	
4§	+	1.0×1.0×1.0	4	−	
5	+	0.3×0.3×0.2	5	−	
6	+	0.3×0.2×0.2	6	+	0.3×0.5×0.3
7	+	0.5×0.3×0.2	7	+	0.2×0.3×0.3
8	−		8	−	

Autopsy was carried out on the 10 th day after implantation.
Each tumor was grey, capsulated, solid and of hard consistence.
* : This was used for passage to treated rats.
§ : These were used for cultivation into tubes.

which were illustrated in Figures 1 and 2 (Plate). No tumors were observed 10 th day and the following days in the controls which received similar amount of amnion primate cells from fresh membranes. No tumors were seen in the radiation controls.

The passages of tumors into next series of conditioned rats were found positive (Fig 5. and 4). It was made by mincing the newly formed tumor of the size 0.5-0.7 cm and transfering in the same manner. Twenty days after implantation tumor nodules were of the size 0.2 to 0.7 cm in diameter in two cases of 5 implanted rats.

The epithelial cells formed tight cluster surrounded with connective tissues in which certain amount of fibroblasts were discerned. This connective tissues did not intrude into parenchym. Each parenchymal cell showed slightly large size and polymorphic shape, of which boundary was sharply defined. The nucleus had considerably large

diameter but generally various sizes. Chromatin content was high. Moderate number of mitosis were observed. No significant histological differences were observed between HeLa-tumor and H-tumor, nor between first generation of H-tumor and its transplanted second generation.

2) Growth pattern of tumor cells in tissue culture.

The difference in the growth curve between original cell lines and the recultivated tumor lines was illustrated in Chart 1. No significant discernible changes were observed. The first passage of the recultivated H-and HeLa-rat-tumor cells revealed smaller size and were coherent. The subsequent passages showed no difference compared with original line.

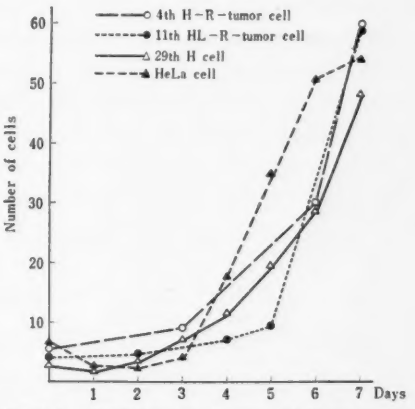

Chart 1. Proliferation Curves of H-rat-tumor Cell and HeLa-rat-tumor Cell Compared with H and HeLa Cells in Vitro.
Growth Medium; 80% LY, 20% Human Sera.

3) Immunocytopathology of H- and HeLa-Rat cells.

Serially subcultivated 11 th H-R cells and 12 th HeLa-R cells in tube cultures with the growth medium were washed with Hank's BSS 3 times and replaced by the 0.8 ml of LY medium containing 5% goat serum. 0.2 ml of undiluted anticellular rabbit serum was introduced into this culture and observations were made for subsequent 5 days.

Preimmunized control rabbit serum series revealed no significant changes by the microscopic examination (Fig. 5 and 6). On the contrary the series with anti-H and anti-HeLa sera against respective cell types revealed profound cytopathogenicity. Granularity in cytoplasm and swelling or contraction of cells were marked. The nuclear changes were striking by enhancement of density of the nucleus with pycnosis. These changes were observed from 20 hours to 5 days.

These changes are illustrated in Figures 7 and 8.

The effect of the anti-HeLa cellular serum on HeLa cells and HeLa-rat tumors were revealed to be in accordance with the findings of McAllister *et al.* (8)

Discussion

The intriguing problem is whether these tumors in conditioned rats derived from established tissue culture cells would disclose the difference between the human tumor and rat tumor, or not. Very recently Moore (9) reported the successful propagation of recultivated human-fibroblast cell-rat tumor into *non-treated* normal rat in certain cases in her screening study on the nature of the tissue culture cell tumor in the

treated rat. It might be indicated that this tumor produced in normal rat would be rat tumor and not human tumor. The changes of the antigenic structure of tumor cells could be attributed to the two major categories, first the selection of mutant cells in the milieu of treated rat, secondly the adaptation or transformation of the internal structure to the rat milieu.

The present study would seem to support the view that the major surface properties of the cells before and after their growth in the treated rat-milieu did not exhibit the difference in the given detectable manner. Therefore it may be assumed that the major population of H and HeLa cells in rats were still human, provided that the technique employed in this test was sensitive enough.

The acquisition of potentiality to propagate itself in the treated rat in the case of "H" strain would be befor 50 passages.

Summary

1) At the Fiftieth passage level established human amniotic cells "H" strain, which was originally derived from normal person, had the potentiality to produce tumors on transfer into the x-irradiated and cortisone treated (Toolan's) rat. Similar results were obtained with HeLa cells.

2) Recultivation of tumors or passage into subsequent series of Toolan's rats were made.

3) Immunocytopathogenic effect of anticellular sera attested to the human nature of tumor cells regrown in the culture with positive results.

The author expresses his grateful thanks to Prof. Hamamoto for his valuable guidance and hearty appreciation to Dr. Tokumaru for his useful advices throughout this study.

He is indebted to Dr. Oda and Dr. Matsuoka for the histological examination in this work.

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Explanation of figures (Plates XIII and XIV)

Fig. 1. H cell tumor in conditioned rat, 1st generation, 10 days after implantation. H-E staining. ×100.

Fig. 2. HeLa cell tumor in conditioned rat, 1st generation, 10 days after implantation. H-E staining. ×100.

Fig. 3. H cell tumor in conditioned rat, 2nd generation, 20 days after transplantation. H-E staining. ×100.

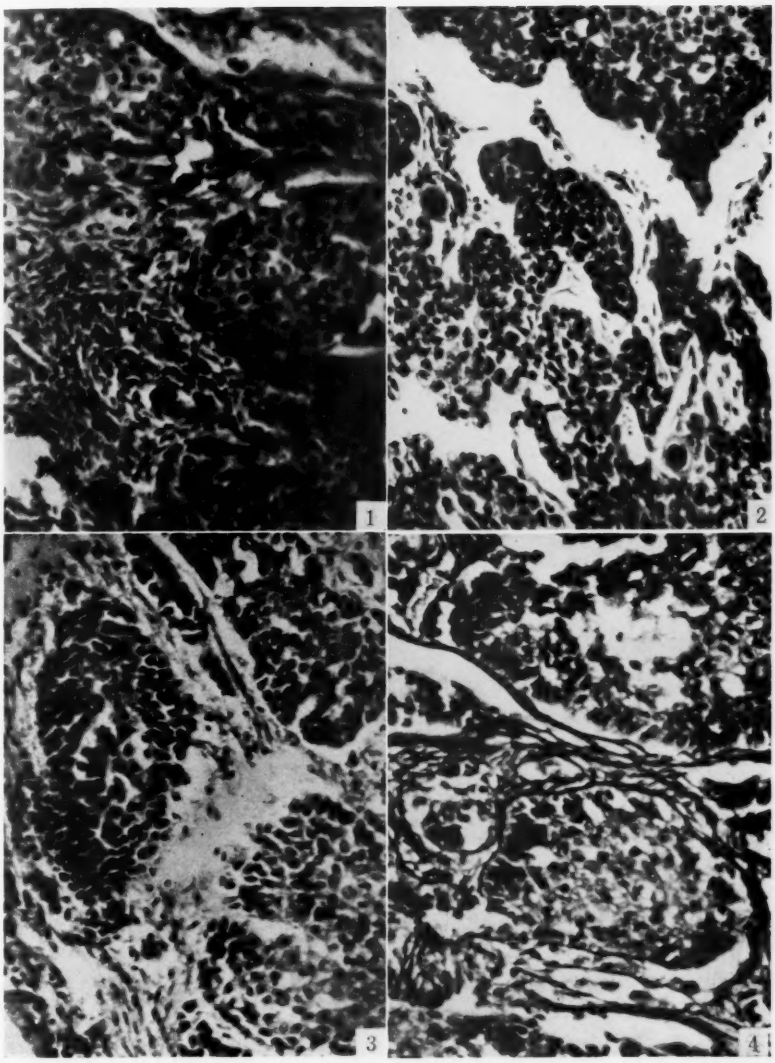
Fig. 4. Same tumor as Fig. 3. Silver staining. ×100.

Fig. 5. 11 th passage generation of H cells in culture, 5 days after adding normal rabbit serum (1 : 5). Giemsa staining. ×50.

Fig. 6. 11 th passage generation of H cells in culture, 5 days after adding anti-H cell rabbit serum (1 : 5). Giemsa staining. ×50.

Fig. 7. 12 th passage generation of HeLa cell in culture, 5 days after adding normal rabbit serum (1 : 5). Giemsa staing. ×50.

Fig. 8. 12 th passage generation of Hela cells in culture, 5 days after adding anti-HeLa rabbit serum (1 : 5). Giemsa staining. ×50.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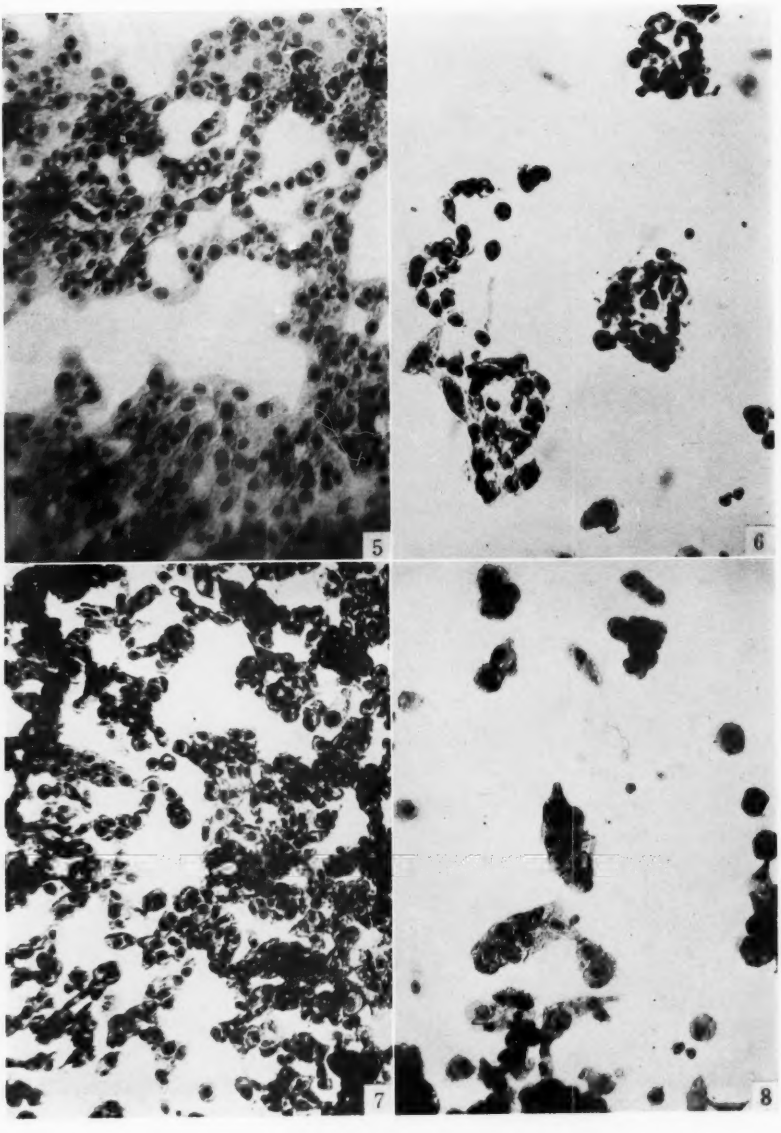
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[GANN, 50, 233~238; September, 1959]

CHROMOSOMAL ALTERATION AND DEVELOPMENT OF TUMORS
II. CHROMOSOME CONDITIONS AND INVASIVENESS OF THE YOSHIDA SARCOMA CELLS[1)]

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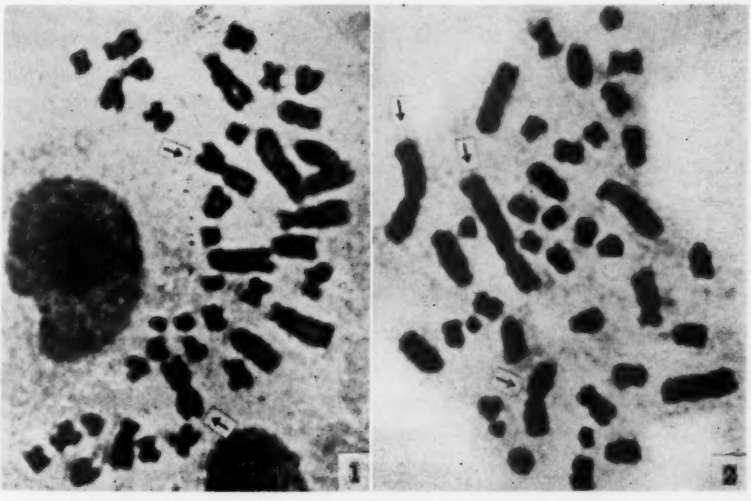
A new subline of the Yoshida sarcoma which is characterized by the presence of three strikingly large chromosomes arose spontaneously in the course of transplantation (Yosida 1957, 1958 a, b). The cells of the Yoshida stock tumor have 40 chromosomes among which two large V-shaped elements are remarkable (Fig. 1), while the new subline possessing also 40 chromosomes, differs from the stock tumor by the existence of a conspicuously large J-shaped element in addition to two large V's (Fig. 2). On the basis of the karyological analysis, it is assumed that the new karyotype was originated by reciprocal translocation (or translocations) between two chromosomes of the original tumor (Yosida 1959).

The tumor cells of the new karyotype increased in number with the increase of



Figs. 1–2. Photomicrographs of metaphasic chromosomes in Yoshida sarcoma cells. 1, cell having an original karyotype. 2, cell having the new karyotype. Arrows indicate the large V- and J-shaped chromosomes characterizing the tumor cells.

1) Contribution from the National Institute of Genetics, Japan, No. 279.

transfer generations. After about the twentieth transfer generation, almost all tumor cells showed the new karyotype (Table 1). At the ninth transplant generation of this sarcoma, the ratio of cells having the new karyotype to the total number of tumor cells was 79.9 per cent in the ascites form.

It is well-known that malignant tumor cells generally invade various organs of the tumor-bearing host. If both kinds of tumor cells, those with the new as well as those with the original karyotype, invaded the organs in the same ratio as that observed in the ascites form, their frequency should be similar. The results of observations showed that the ratio of both karyotypes in the organs was quite different from that in ascites form and strikingly varied accordingly to the organs examined.

The present paper deals with the results of some cytological observations with special reference to the invading ability of tumor cells with different chromosome constitutions, and describes the technique for study of tumor cells invading various organs.

The author wishes to acknowledge his indebtedness to professor Sajiro Makino, Hokkaido University, for his keen interest in this investigation and his kind revision of the manuscript.

Technique

The stock line of the Yoshida sarcoma from which the new subline was derived was obtained from the Institute for Infectionus Diseases, Tokyo. The tumor was transferred serially from rat to rat in the National Institute of Genetics, Misima (for details, see Yosida 1959).

To observe the chromosomes in invading tumor cells is difficult because of the small number of tumor cells. Since a large number of mitotic cells is needed for their statistical study, small pieces of various organs containing tumor cells were removed from tumor-bearing rats and inserted into peritoneal cavities of new hosts. This method made possible a proliferation of tumor cells in the peritoneal cavity forming a new cell-population in ascites form. In this way, three ascites populations in the tenth transfer generation were obtained from inoculations of liver, lung and spleen of a tumor-bearing rat in the ninth transfer generation (Fig. 3). On the other hand, a tumor mass of the Yoshida sarcoma has developed in the peritoneal cavity of the same tumor-bearing rat mentioned above. By inoculation of small pieces of the tumor mass into the peritoneal cavities of new hosts a cell population of ascites form newly developed. As control experiment, ascites tumor cells were transferred into intraperitoneal cavities of new hosts by usual transfer technique.

A practical method for organ transplantation may be given as follows: Small pieces of liver, lung, spleen and a tumor mass of a tumor-bearing rat were washed three or four times with normal saline containing peniciline and streptomycin in order to wash down the tumor cells adherent to the surface of the organs as completely as

possible. Then small pieces of the organs were inserted into the peritoneal cavities of non-tumorous animals with the aid of a tradsplantation needle.

Ascites tumor cells were observed by acetic orcein squash technique after treatment with hypotonic solution.

Results of Observations

1) *Control* (observations in the ascites tumor cells developed by usual transfer technique): Observations at the ninth transfer generation of the ascites tumor of a usual form showed that ratio of the new karytype cells to the total number of tumor cells was 82.6 per cent. The data shown in Table 1 and Fig. 3 indicate that the ratio of the new tumor cells gradually increased with the increase of transfer generations. This seems to imply that the tumor cells of the new karyotype are superior in adaptability to the original cells.

2) *Observation in ascites tumors obtained from the inoculation of a solid tumor:* A small piece of tumor mass which developed in the tumor-animal was inserted into

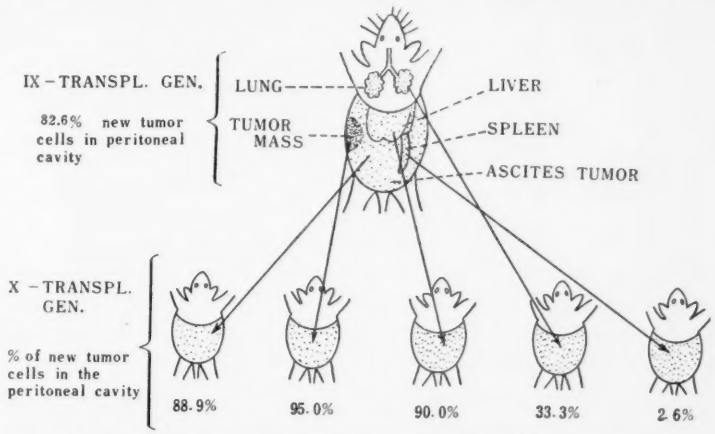


Fig. 3. Scheme of organ-transplantation and incidence of tumor cells having a new karyotype.

peritoneal cavities of new rats. Out of three rats thus treated, two developed an ascites tumor in their peritoneal cavities. The karyological examinations of these tumors showed that 95 per cent of tumor cells under study represented the new karyotype. The results show that the vast majority of tumor cells forming the solid tumor used for inoculation were of the new karyotype.

3) *Observations of ascites tumors induced by liver inoculation:* Two or three pieces of liver tissue derived from a tumor-bearing animal were inoculated into peritoneal cavities of three non-tumorous rats, one of which developed an ascites tumor. It is most probable that the ascites tumor thus produced arose by proliferation of

Table 1. Occurrence of the new karyotype in several transfer generations of the Yoshida sarcoma.

Transfer gener.	No. of cells of new karyotype	No. of cells of original karyotype	No. of cells observed	% of new karyotype
I	0	25	25	0
V	5	3	8	62.5
VI	12	17	29	41.4
VII	37	24	61	60.7
IX	119	25	144	82.6
X	96	12	108	88.9
XI	106	30	136	77.9
XIII	61	7	68	89.7
XV	99	11	110	90.0
XX	185	0	185	100.0
Total	720	154	874	

tumor cells which invaded the liver. 90 per cent cells in this tumor showed the new karyotype, while about 10 per cent were of the original karyotype.

Table 2. Frequency of tumor cells having the new karyotype in ascites tumor which developed by organ transplantation.

Organ	No. of new tumor cells	No. of original tumor cells	Total no. of cells observed
Solid tumor	38(95%)	2	40
Lung	15(33.3%)	30	45
Spleen	1(2.6%)	38	39
Liver	36(90%)	4	40

4) *Observations of ascites tumor induced by lung-and spleen-inoculation:* Small pieces of lung and spleen tissues were inoculated separately into peritoneal cavities of 5 rats. One rat with the lung-inoulation and another with the spleen-inoculation developed ascites tumors. The karyological study of cells of those tumors produced the surprising result that 33.3 per cent of tumor cells from lung-inoculation, and 2.6 per cent from spleen-inoculation were of the new karyotype. Bassed on these results, it is reasonable to conclude that the majority of tumor cells which invaded the lung and the spleen had the original karyotype.

Remarks

Invasion of organs of tumor-bearing animals by tumor cells is a subject of primary importance in cancer cytology. It was found that malignant cells can invade any place in the body through transportation by the blood system. Yoshida (1949) reported that the Yoshida sarcoma infiltrates every organ of the tumor-bearing rat.

Observations of the chromosome condition of the invading tumor cells indicate that the success of inoculation seems to differ according to their chromosomal pattern. It seems that tumor cells with the new karyotype show a predominant peritoneal proliferation in an ascites or a solid form and a particular adaptability to liver tissue, while those having the original karyotype are well-adapted to spleen and lung tissues.

In the chromosomal studies of human cancers Yosida and Tabata (1958) and Tabata (1959) reported that the majority of the tumor cells of the primary stomach cancer had hypotriploid and hypopentaploid chromosome constitutions, whereas the tumor cells of the maxillary tumor which was based on a metastasis of stomach cancer showed a hypodiploid chromosome constitution. The evidence of the difference in chromosome pattern between the maxillary and the primary stomach cancer indicates that a selective adaptation takes place in metastatic cells. A similar concept of selecitive adaptaptation of tumor cells could be applied in the case of the Yoshida sarcoma involving the difference in adaptability of tumor cells to particuler tissues.

The relationship between the chromosomal pattern of tumor cells to their infiltration-ability may be important in connection with the problem of metastasis.

Summary

The results of observations of chromosomes in tumor cells invading various organs of a Yoshida sarcoma rat were described in the present paper.

New tumor cells of the Yoshida sarcoma which possess a prominently large J-shaped element have developed in the course of transplantations at Misima. These tumor cells gradually increased in number during the course of ensuing generations. In the process of establishing the new tumor subline two types of cells, with the new and the original karyotypes, were observed. Using this tumor material, the facility of invasion of various organs by the tumor cells with two kinds of karyotypes, the new and the original, was examined. The results of observations indicate that infiltration-ability of tumor cells into tissue differs according to their chromosomal pattern. The tumor cells with the new karyotype showed a predominant peritoneal proliferation in an ascites or solid form and a particular adaptability to liver tissue, while those having the original karyotype were well-adapted to spleen and lung tissues.

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[GANN, 50, 239～249; Septembr, 1959]

CYTOLOGICAL STUDIES OF NEOPLASM. V. MORPHOGENESIS OF V-CHROMOSOME IN YOSHIDA SARCOMA CELLS WITH SPECIAL REFERENCE TO ITS STRUCTURAL ABERRATION

(Plate XV)

YUZO MARUYAMA

(Department of Pathology, Faculty of Medicine, Shinshu University. Director: Prof. Dr. Z. Ishii)

Makino and his co-workers demonstrated, by cyto-karyological study of many experimental ascites tumors of rats and mice, that each tumor maintained its own characteristics concerning distribution of chromosome (abbreviated to chrm.) number and chrm. constitution. Such properties are preserved by the proliferating group of tumor cells called "stemline cell" and also found in the successive cell generations of the stemline cells (Makino[1)2)], Makino and Kanô[3)]). The "stemline theory" was reapproved by Hauschka[4)] and Maruyama[5)] and is now supported by many investigators.

For karyological determination of a stemline, V- or J-chrm. with prominent size can offer an useful criterion, because the chrm. constitution of host somatic cells is devoid of such chrm. (Tanaka[26)], etc.). The V-chrm. was discovered in Yoshida sarcoma cells by Makino[3)6)], and the V- or J-chrm. have been found in various tumors of rats and mice thereafter; in Hirosaki sarcoma[7)], Yoshida ascites hepatoma[5)16)29)], Makino's ascites hepatoma[9)], Watanabe ascites hepatoma[10)], Usubuchi sarcoma[8)], MTK-sarcoma[11)], Takeda sarcoma[12)] in rats and Takizawa quinonecarcinoma and Ehrlich's carcinoma[13)] in mice. Hirosaki sarcoma was divided into 5 sublines by Kanô[7)] on the basis of the different appearance of V-chrm. Recently Makino[11)14)] and Tsuda[15)] found the derivatives from the original stockline of Yoshida sarcoma showing distinguishable features of chrm. constitution, especially of the V-chrm. Maruyama[5),16)] also confirmed the characteristically increased V-chrm. number in hemorrhagic subline of Yoshida sarcoma. Many other studies revealed the aberrated morphological features of chrm. member in malignant tumor cells, but it is considered by many investigators that the characteristic V- or J-chrm. is rather stable for each tumor line. As the morphogenesis of these characteristic V-chrm., T.H. Yoshida[17)] reported that the two V. chrms. in Yoshida sarcoma originated from central fusion of 6th and 7th, and 8th and 9th chrms. respectively, and that the largest V-chrm. in Hirosaki sarcoma arose from terminal fusion of 2nd J- and 6th R-chrm.

The purpose of the present study consists in the reinvestigation of chrm. constitution of the original stockline of Yoshida sarcoma in order to analyze the morphogenesis of V-chrm.

Material and Method

The observation was performed by the stockline (2V-type) of Yoshida sarcoma used in the previous study[6]; ascites accumulated in 3rd day after inoculation was obtained from 2 rats. The technique employed was essentially similar to that described by Makino *et al*[18]: a drop of destilled water was added to a drop of ascites, then remained for 2-5 min. on slide, stained subsequently by aceto-orcein in the ordinary way, finally the coverslip was slightly pressed down for making chrm. disperse and sealed with paraffin-balsam.

The analysis of chrm. members was performed by microphotographs of metapase plate, from which serial alignments of chrm. members were redrawn. It is impossible to determine the actual length of a chrm., because the degree of chrm. contraction is different in each mitotic stage and spindle chrm. mechanism is also disharmonized.

Consequently, for the purpose of quantitative com parison of chrm. length, the 1st longest J-chrm. was standarized as 30 in length; those of chrm. or its arms are then expressed in figures as a relative value to the 1st J-chrm.

Observations

12 (ser. 1) and 70 (ser. 2) microphotographs of metaphases taken from the ascites obtained on two occassions from two rats were available. Their chrm. numbers are given in Fig. 1, where the modal variation is confined within a subdiploid range. Cells with 40 chrms. are most commonly encountered, their frequency being 6 in 12 (ser. 1) and 31 in 70 cells (ser. 2). The characteristic V-chrm. with prominent size are counted 2 in one cell in high percentage in both series, being 11 in 12 (ser. 1) and 61 in 68 cells (ser. 2) except 2 tetraploid cells in ser. 2. Only one V-chrm. is encountered in the other cells. Therefore the number of the prominent V-chrm. per cell is relatively constant. But the V-chrms. are found in wide aberration in relative length between two arms, size, bending at the 1st constriction and so on.

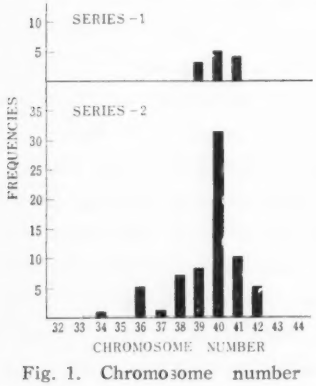

Fig. 1. Chromosome number distribution.

Several types are illustrated in Fig. 2. Types A-G, and another type, named Type H must be added. They are schematically shown in Fig. 3.

1) *Type A.* It consists of two curved arms; the one is longer and more highly

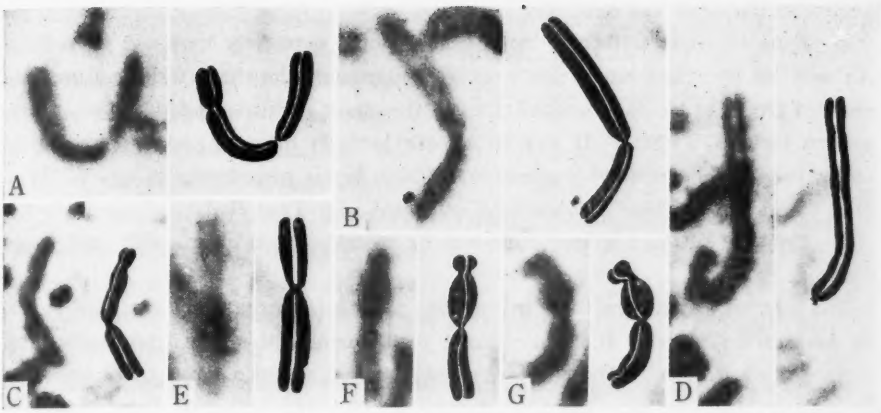

Fig. 2. Various types of V-chromosome oberved in Yoshida sarcoma cells. (photomicrographs and their illustrations)

curved than the other, with an appearance of U or γ. They are found to be curved gently but irregularly in two cells. Their relative length is 20-23 in the long arms and 15-20 in the short arms, the ratio is approximately 1.2-2.0. The long arm has often a 2nd constriction at its distal portion. It is found infrequently in the short arm too. This type, when appeared within a cell, is only one invariably, and is observed in 2 in ser. 1 and 9 cells in ser. 2, in combination with Type H in 9 and with Type E in 2 cells.

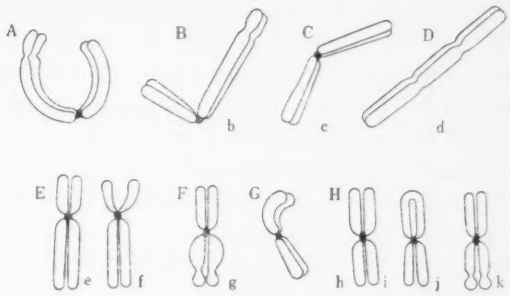

Fig. 3. Schematic drawing of the modal types A-H V-chromosomes Type H has occasinally a closed end (j) or a 2nd constriction on one arm (k).

2) *Type B.* It is subtelocentric chrm. consisting of two arms with clear difference in length and is bended at the 1st constriction. Relative length of the arms are 20-28 in the long and 10-15 (17 in one cell in extreme) in the short arms. This type, when appeared, is always one within a cell as Type A and is observed in 2 and 12 cells in the two series respectively, in combination with Type H except one case combined with Type F.

3) *Type C.* It is nearly metacentric chrm. consisting of two strait arms forming a bending at the 1st constriction. Its arms appear narrow in width and it is difficult to recognize the separation of chromatids. The relative length of the arms is approximately 12-16 in both. This type is observed in 1 and 5 cells in the two series respectively in combination with Type H in 3 and with Type E or G in 1 cell res-

pectively, the other one remaining alone.

4) *Type D.* It is neither V- nor J-chrm., but is extremely long and telocentric. As will be discussed later, this type also represents the characteristic aberration resulted from the similar mechanism as in the other V-chrms. and this type is considered here as V-chrm. It has no smooth surface, indicating some isthmi in its course (Fig. 2). Its relative length is 44 and 38 in 2 cells respectively in ser. 2. Type D is found only in these 2 cases, both combined with Type H.

5) *Type E.* It is J-shaped submeta- or subtelo-centric chrm. with two arms differing in length.

The relative lengths are 14–19 in the long and 10–14 in the short arm, but varied to some extent making it often difficult to distinguish this type from the smaller types described below. The both arms are arranged strait in general, but some are irregularly bent. In some cases are formed the 2nd constriction at distal portion of both arms. This type is observed in 17 cells in only ser. 2, 15 of which are combined with Type H, one with Type G and the remaining is found alone.

6) *Type F.* Types F, G, and H are approximately similar in size and metacentric being classified into the small group; the relative lengths of the arms are 10–16 in general but varied in wider range than in the other types.

Type F is morphologically characteristic, showing a swelling in one arm with an appearance of ninepins and with a 2nd constriction at distal portion. Such an appearance seems to come from loosened spiralization of chromonema. This type is observed in 2 and 3 cells in the two series respectively, always in combination with Type H.

7) *Type G.* It is characterized also in morphology by a hooked sharp bending on one arm. It is not clearly proved whether it is a well established type or a temporary phase of chrm. contraction caused by abnormal spiralization of chromonema. This type is observed in 7 cells through the two series, in combination with Type H in 3, with Types C, E, and G in one cell each and the remaining one is found alone.

8) *Type H.* It is the V-chrm. most commonly to be encountered; it appears in 73 cells (89.0%) through the two series, 16 of which contained Type H only, and 6 among them only one of this type. The other 57 cells are in combination with the other types as already described.

Type H is a member of the small class (Types F, G, H), but has no morphological characteristics as Type F or G. The small class varies in its size within a wider range than those of the other types, especially in Type H, and this fact suggests that there may be presumably found some modal types among the small classes especially in Type H.

Viewed from chrm. size, the V-chrms. are classified into three; the prominent

(Types A–D), the intermediate (Type E) and the small class (Types F–H) (Fig. 3). Combinations of the various types of V-chrm. found within a cell are shown in Table 1 collectively.

The prominent V-chrm., when it is found in a cell, is always single, neither plural in number nor coexsists with the other prominent V-chrm., whereas the intermediate or small classes are observed frequently plural in number. It is therefore suggestive that the prominent V-chrm. is essentially different from the other classes.

A								
B								
C								
D								
E	2	1	1					
F								
G			1		1		1	
H	9	11	3	2	15	5	3	10
	A	B	C	D	E	F	G	H

1–V { C–1, E–1, G–1, H–6.

Table 1. Combination of V-chromosomes within a cell, 1-V cells are listed separately.

The serial alignments obtained by standarization of the length of the 1st J-chrm. as 30 are shown in Fig. 4; the chrm. constitutions in different nuclear plates or idiograms varied in minor range, but it is possible to determine or identify accurately each chrm. member in approximately the half of the total idiograms. A Y-chrm. is

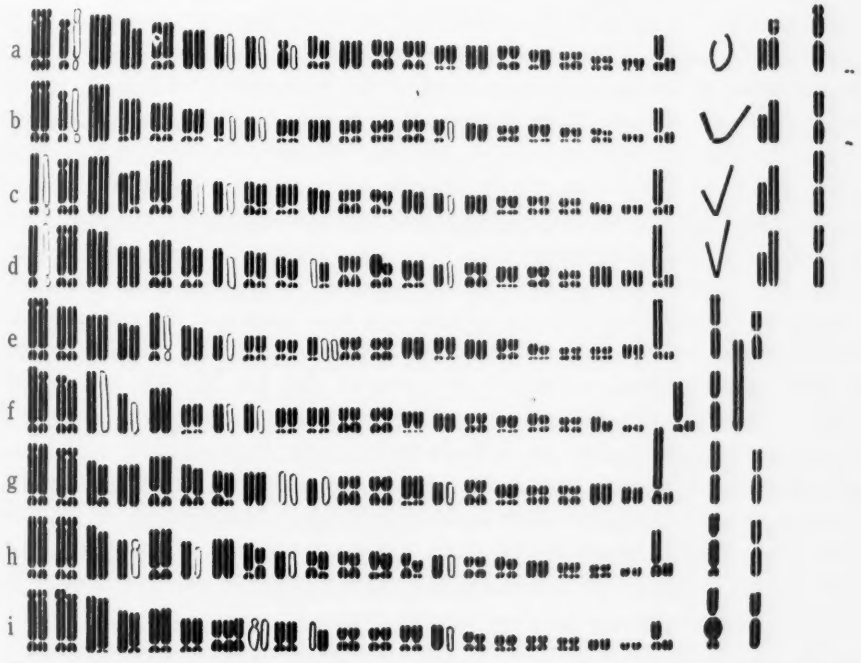

Fig. 4. Serial alignments of chromosomes —— a, b (Type A, H): c, d (Type B, H): e (Type C, H): f (Type D, H): g, h (Type E, H): i (Type F, H).
Each alignment is consisted of 40 and 41 (e, i) chromosomes, two of which represent the characteristic V-chromosome shown at the end on the right side.

easily identifiable, suggesting that Yoshida sarcoma cell is male in origin as described early by T.H. Yosida. But the 6-10th or -11th chrm. moderate in size and telo- and subtelo-centric, can not find their proper positions in idiogram because of their variations in size and shape. In general, the smaller members are not relatively so decreased in length in case of chrm. contraction in lower degree.

In Fig. 4-a, the 2nd, 7th, 8th and 9th chrm. sets are unpaired, lacking in their companions (indicated as hollow chrm); in Fig. 4-b, 2nd, 7th, 8th and 14th chrm. sets are unpaired. In both cases, the 2nd large J-chrm. is destitute of the companion. This phenomenon is found through all nuclear plates with Type A chrm. The 2nd J-chrm. appears often in curved condition in its long arm, which is entirely equal in length to the long arm of Type A-V-chrm. 20-28 long. It is suggested from this fact that Type A chrm. is originated from terminal fusion between distal end of the short arm of the 2nd J-chrm. and central part of moderate sized R-chrm., whose relative length is estimated as 10-15. In the same way, in Fig. 4-c, 1st, 6th, 7th and 14th chrms. are unpaired, and in Fig. 4-d 1st, 7th, 10th and 14th chrms. too. The 1st large J-chrm. is destitute of a companion not only in the last two cases but also in all idiograms with Type B-V-chrm. and its strait long arm corresponds to the long arm of Type B-V-chrm. in length. This suggests that Type B is originated from terminal fusion between terminal end of the short arm of 1st J-chrm. and the central part of a member of the moderate sized chrms. Fig. 4-e is the idiogram with a Type C-V-chrm.; here are unpaired 5th, 7th, 10th and 14th chrms. but the other plates with Type C often destitute of 4th chrm. companion in place of 4th. The 4th and 5th pairs are not clearly determined as to their positions in the idiogram, but it is to be noted that the destitute chrms. are always J-chrm. It is suggested from the result that Type C is originated from terminal fusion between distal end of the short arm of 4th or 5th J-chrm. and central part of a member of the moderate sized chrms. In Fig. 4-f, the idiogram with a Type D-V-chrm., 3rd, 5th, 7th and 8th pairs are destitute of their companions. It is suggested also that Type D is originated from terminal fusion between distal end of 3rd large R-chrm. (30 in length) and central part of a member of the moderate sized chrms. (14 in length), forming an extremely long telocentric chrm. Fig. 4-g and Fig. 4-h are the idiograms with Types E and H, and Fig. 4-i those with Types F and H; the destitute chrms. companions are found in 5th-9th or-10th pairs, which are not also accurately determined as to their positions. The central fusion in Type E-Type H may be caused by the moderate sized R-chrm. in the undetermined groups.

It is further suggested that the one chrm. component forming each of the prominent class (Types: A-D) is also originated from the group just mentioned as in the case of Type F-Type H (Fig. 5). The relative length of lhe component is measured as follows: 10-15 in Type A, 5-8 in Type A, 5-8 in Type B, 8-12 in Type C, 8-14 in

Type D. Therefore it is quite possible that the moderate sized R-chrm. is combined with all types of V-chrms by fusing in the central portion. It is observed, on the other hand, that chrm. members in the moderate sized group are greatly aberrated in morphology in general.

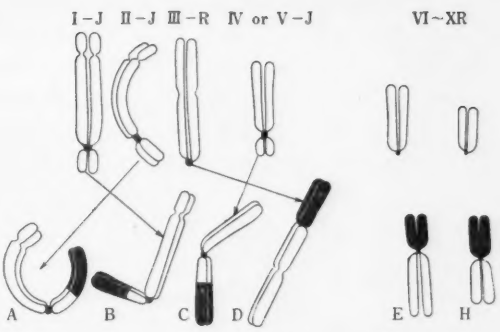

Fig. 5 Schematic explanation on morphogenesis of V-chromosome. The affected chromosomes (dark) form the various types of V-chromosome as the result of fusion with the other chromosomes.

As it is possible to find all types of V-chrm. among the tumor cells, it is difficult to conclude that V-chrm. especially the prominent class is merely an aberrative or degenerative change in chrm. constitution caused by abnormal mitotic cours. Recently Levan[19] suggested "interchromosomal translocation" for the chrm. aberration from the practical measurement of the chrm. length in mouse ascites tumor cells. The morphogenesis of V-chrm. as the result of a simple terminal fusion presumably caused by a certain damage, may be also interpreted in the analogous sense as Levan's hypothesis.

Discussion

It has been thought the V-chrm. in Yoshida sarcoma cells are invariable in size and shape and derived from central fusion. The author found several types of V-chrm. different in size and shape and concluded that they are often derived from terminal fusion. The chrm. constitutions are reciprocally varied in minor range, but the majority of chrm. members is always clearly identified and is also found in normal rats; the variations of V-chrm therefore can not be thought as artefacts. Among the chrm. members particularly varied in shape or size are the 6th-9th chrm. The prominent V-chrm. in Yoshida sarcoma has been studied from various aspects by many; the original stock line with a simple V-chrm. or some sublines with two or more or a cell with two V-and one large J-chrm. Such chrms. are all nearly metacentric and moderate in size; they are described to be drived from 6th-9th telocentric chrms. by central fusion (T.H. Yosida). The V-chrms. observed by the author are varied widely and considered presumably to be derived from 1st -3rd chrm. by fusion of the other moderate sized chrm. The varied V-, J- or R-chrms. are also found in the other experimental tumors of rats (Fig. 6); the largest V-chrm. in Hirosaki sarcoma was derived from fusion of 2nd large J- and 6th moderate sized R-chrm by T.H. Yosida, which can be thought as Type A. V-chrm. observed in MTK-IV

Fig. 6. V-chromosome in various tumors of rats Yoshida sarcoma (a-d), Hirosaki sarcoma (e-f), MTK-IV sarcoma (g), Watanabe ascites hepatoma (h), Takeda sarcoma (i), Usubuch sarcoma (j), Azo-dye-treated hepatoma H-1b and W-1 (k-1).

sarcoma (Fig. 6-g) (Makino[11]), in Watanabe ascites hepatoma (Fig. 6-h) (Watanabe[10]), in azo-dye-treated hepatoma H-1b (Fig. 6-k) (T.H. Yoshida[24]) and in Murphy's lymphosarcoma T.H. (Yosida[23]) also belong to Type A. Type B is seen in azo-dye-treated hepatoma H-2, and W-1 (Fig. 6-l) (Yosida, T.H.[24]), Type C in Walker carcinosarcoma (T. H. Yosida[23]), and Type D in Usubuchi sarcoma (Fig. 6-j) (Makino[11]). In the last case, an extremy long subtelocentric chrm. can be seen, which is also considered to be derived from terminal fusion of 1st or 3rd chrm. with the moderate sized chrm.

From the above considerations, it is reasonable to think that the prominet V-chrms. are confined to Types A-D and completely differed from those of mouse tumor cells. They are essentially of the same origin and of the same morphogenesis. The intermediate or the small class, though not explained in detail, is interpreted in the same way. The karyological characteristics of a tumor line should be based upon the characteristic combinations of the modal patterns of V-chrms.

It has been believed that the appearance of the prominent V-chrms. characterized the chrm. constitution of a tumor stemline, as in case of Yoshida sarcoma; it is stable in occurrence and feature for karyological determination and the other intermediate or small class is developed as the result of deviation of a stemline of abnormal conditions through decreased vitality or denegeration. This view not correspond to the author's result apparently.

The author postulates here the morphogenesis of V-chrm. as follows: it occurs primarily as the lesion or breakage in a specific portion of a specific chrm. and then various types of V-chrm. are developed by fusion of the two chrms. If the frequency of V-chrm. is determined by the affected chrm. number, it will be well understood

that it preserves constant value relatively against the wide variation of V-chrm.

It is found recently by Tjio and Oestergren[27] that the virus-induced mammary carcinoma cells of mouse (C3H, DBR) have the pycnotic chrm. just like a sex chrm. resulting from combination with virus, which seems essential for malignancy. The V-chrm. observed in many tumors of mouse and rat can not be seen here. This hypothesis suggests that a single chrm. member can be altered by malignant change. The conditions of moderate sized chrms. are thought to be analogous to the pycnotic chrm. above described. Hence it is concluded that central damage of the moderate sized chrms., viz. chrm. mutation, may be essential for malignant change, and the characteristic V-chrms. various in shape and size, are secondarily derived from fusion between the two special chrms. From this point of view, it is very interesting that DAB-hepatoma cells have various types of V-chrm. in its early stage of development (T.H. Yosida).

Summary

The chrm. constitution of Yoshida sarcoma cells was observed by the water-pretreatment method and was analysed on microphotographs. The chrm. numbers were 40 in common generally and were in subdiploid range with few exceptions. The prominent V-chrm. with a characteristic appearance, are found 2 in a tumor cell in high percentage and constancy. But they varied in structure and are classified into several types: Type A consists of 2 curved long arms, the longer arm of which is originated from 2nd J-chrm.; Type B is rather J-shaped with a straite short arm and a long arm, the latter originating from 1st J-chrm.; Type C is bent at 1st constriction with 2 approximately equal arms, one of which is originated from 5th J-chrm.; Type D consists of an extremely long telocentric chrm. and a short arm, the former originating from 3rd telocentric chrm.; Type E is a submetacentric chrm. intermediate in size; Types F, G and H are similar in size and in relationship between the 2 arms, representing the small class of V-chrm. Types F and G are characterized by the peculiar formation on one arm, and distinguished from Type H. Type H is most common to be encountered. It is determined without difficulties as one component of chrm. in the prominent V-chrm., but its companion can not be determined immediately. It is also determined with uncertainty both components of V-chrms. other than the prominent V. Those chrm. characterized by their moderate size and telocentricity, are found as a companion of a V-chrm. in any type invariably, though it is difficult to identify each of them as a companion. Centric fusion of these chrms. has been assumed by many in regard to the chrm. constitution of the rat tumor cells.

The author is different from others in opinion and offers a hypothesis as follows: the central damage of moderate sized chrm., viz. a short of chrm. mutation, is essential for malignant cellular change; these chrms. are structurally widely varied through

selection of a companion or of modus of fusion, central or terminal, and the number of the affected moderate sized chrms., will determine the number of the characteristic V-chrms. against the wide morphological variations.

I wish to express my thanks to Prof. Dr. Z. Ishii for his advice and encouragement throughout this work. Thanks are also due to Prof. Dr. S. Makino and Dr. M. Sasaki of Hokkaido University and Dr. T.H. Yosida of The National Institute of Genetics for their interests in my work and giving helpful direction on the procedure.

Explanation of Plate XV

Micropfotographs of nuclear plates: a, b (Type A, H), c, d (Type B, H), e (Type C, H), d (Type D, H), f (Type F, H), g (Type G, G).

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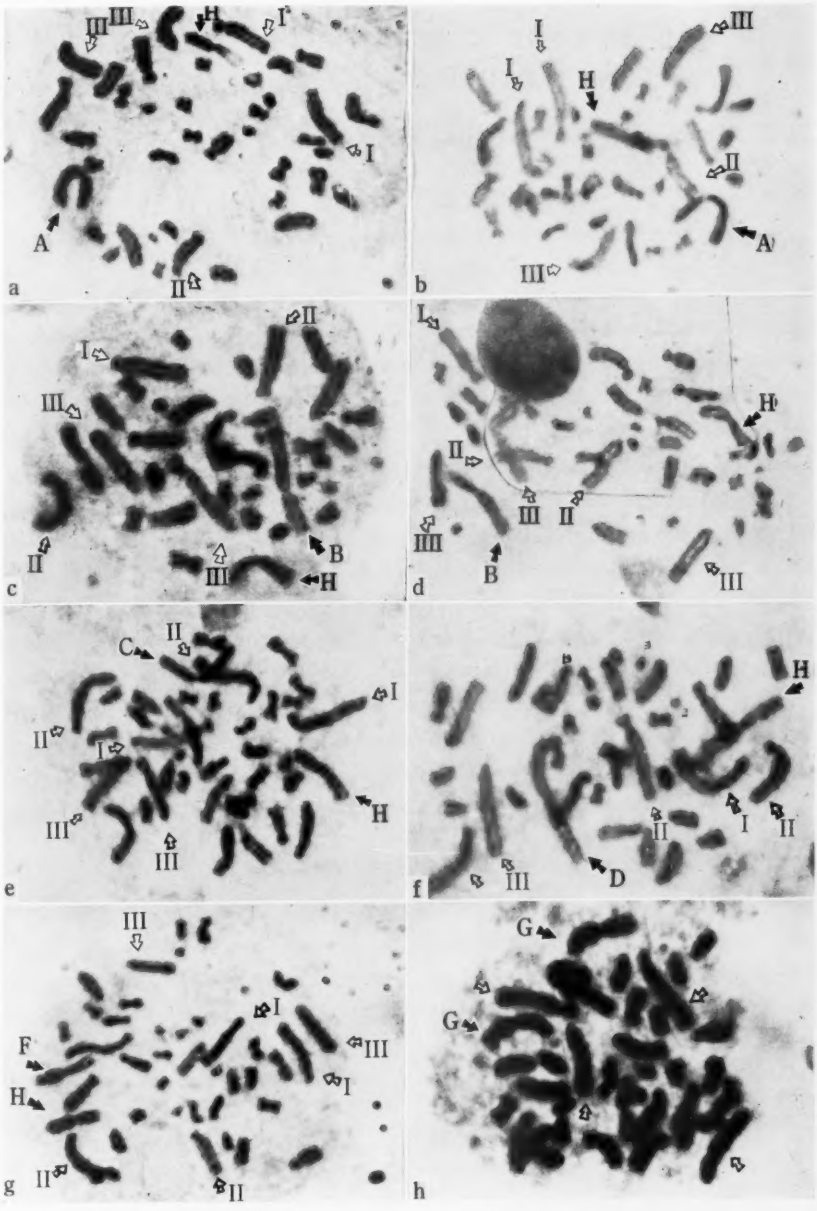
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[GANN, 50, 251~264; Septenber, 1959]

EFFECTS OF COMPOUNDS ON THE FRIEND MOUSE VIRUS LEUKEMIA*

(Plates XVI–XVIII)

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Although as yet there is no evidence that human cancers are caused by viruses, much experimental evidence indicates that viruses may be the cause of neoplastic growth. If viruses are a significant factor in human cancer, then finding a cure for cancer may be simplified.

About four years ago Dr. Charlotte Friend discovered a new transplantable mouse leukemia (1) (Fig. 1). This leukemia is caused by a virus as indicated by somes of the following characteristics. The agent is transmissible in adult mice by means of cell-free filtrates of the leukemic spleen homogenates. Splenic cellular suspensions subjected to massive doses of X-rays (100,000 r) are still infective. Filtrates remain stable when stored at −75°C or when lyophilized. The agent is inactivated by heating to 56°C for 30 minutes and by exposure to ether or formalin.

About two years ago this new transplantable mouse virus leukemia was added to our spectrum of tumors. It was of interest to determine tne responses to various chemotherapeutic agents of a malignancy known to be caused by a virus.

Materials and Methods

The present report represents a preliminary study in which a possible screening procedure was explored. It consisted of seven daily intraperitoneal injections of compounds at maximum tolerated doses given 1 to 7 days after intraperitoneal injection into mice (Swiss albino mice, female, 18–22 g) of 0.2 cc of a 10 per cent saline homogenate of leukemic spleens. This transplanted leukemia kills mice in 2 to 4 months with marked enlargement of liver and spleen. The animals were

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A portion of this material was presented at the Seventh International Cancer Congress, London, July 6–12, 1958.

maintained on a standard pellet diet (Purina Laboratory Chow) and water *ad libitum*. As an initial approach, the effects of the compounds were evaluated by comparing the weights of spleens in treated and untreated infected mice 3 weeks after intraperitoneal injection of the leukemic spleen homogenates. For screening purposes this end point was selected in place of survival time because of the 2 to 4 months' observation period that the latter would require. Supplemental information is being obtained for those compounds showing marked or complete inhibition of the increase in spleen weight. This includes titration of virus in those spleens and determinations of increase in survival time from treatment with the compounds passing the initial screen.

The injection volumes of the solvents used for the chemotherapeutic agents were as follows: saline (S), 0.5 cc; carboxymethycellulose (CMC), 0.5 cc; propylene glycol (PG), 0.5 cc; alcohol and saline (A and S), 0.5 cc; peanut oil (PO), 0.1 cc; all once daily; 0.1 to 0.2 cc for all subcutaneous treatments.

All compounds except steroids were injected intraperitoneally. Cortisone and other steroids were injected subcutaneously.

The degree of inhibition of virus leukemia was graded according to the following scheme:

− No effect (spleen weight from three quarters or more of the average spleen weight of the leukemic spleens of the controls).

± Slight inhibition (spleen weight from one half to three quarters of the average spleen weight of the controls).

+ Moderate inhibition (spleen weight from one fourth to one half of the average spleen weight of the controls).

+‡ Marked inhibition (spleen weight approximatety one fifth of the average spleen weight of the controls).

‡ Complete inhibition (no alteration of spleen weight from normal, nonleukemic animals).

Spleens of mice three weeks after intraperitoneal injection of 0.2 cc of a 10 per cent leukemic spleen homogenate weighed from 0.5 to 5.0 g but generally around 2 g, while spleens of normal mice (about 8 weeks old) weighed from 0.16 to 0.4 g, but generally 0.2 g or less.

Results

The results obtained with compounds tested at or near maximum tolerated doses are summarized in Tables 1 to 5. Compounds studied included alkylating agents, antimetabolites, antibiotics, steroids, substituted amino acids, and miscellaneous compounds. Each compound was tested using 5 to 10 mice bearing the virus leukemia, and experiments were repeated in many cases.

Table 1. Compounds Inactive Against Friend Virus Leukemia

Compound	Solvent	Dose mg/kg/day	Av. spleen weight at 3rd week (g) treated/controls*	Tumor index*
Colchicine	S	5.0	1.48/1.88	0.83
N-Methyl acetamide	PG	1,500	1.91/1.69	1.13
5-Bromouracil	CMC	500	2.35/2.88	0.82
5-Fluorodeoxyuridine	S	40	1.58/1.87	0.84
S-Triazolo(4, 3-d)pyrimidine	S	10	3.97/2.17	1.83
3, 3-Diethyl-1-phenyltriazene	PO	35	1.55/1.88	0.83
N-(p-Fluorophenacyl)-2-bromo-pyridinium bromide	S	10	1.30/1.51	0.90
2-Amino-5-nitrothiazole	CMC	75	1.14/1.40	0.81
2-Ethylamino-1, 3, 4-thiadazole·HCl	S	37.5	1.62/1.62	1.00
5-Methyl-2, 1-arabo-benzimidazole	CMC	250	1.31/1.49	0.90
2-Allylimino-1, 3, 4-thiadiazoline·HCl	S	100	2.27/1.69	1.34
2-Ethyl-4-nitroquinoline-N-oxide	CMC	8.0	1.57/1.67	0.94
Streptomycin	S	500	2.00/2.20	0.91
Chloramphenicol	CMC	500	2.20/2.64	0.83
6-Diazo-5-oxo-*L*-norleucine	S	0.05	1.63/1.63	1.00
Actidione	S	12.5	1.57/1.63	0.96
Fumagillin	CMC	10	1.61/1.38	1.17
Actinomycin D	A and S	0.025	1.93/1.46	1.32
Sarkomycin	S	250	2.67/3.43	0.78
Carzinophilin	S	2,500 uits	1.76/1.93	0.91
Kanamycin	S	500	1.88/1.94	0.96
Sulfocidin	A and S	0.2	1.91/1.79	1.09
Δ^4-Androsten-9α-fluoro-17-methyl-11β, 17-β-diol-3-one	CMC	375	2.98/1.47	2.03
2, 4,6-tris(*o*-Hydroxyphenyl)-*s*-trithiane	CMC	15	1.86/2.03	0.92
3-[2-(3, 5-Dimethyl-5-acetoxy-2-oxo-cyclohexyl)-2-acetoxyethyl]-glutaramide	S	0.5	1.70/2.03	0.84
d-Parasidol	CMC	100	1.49/1.93	0.77
l-Parasidol	CMC	100	1.45/1.93	0.76
Lutrexin	CMC	500	1.59/1.87	0.85
Methylene blue	S	30	2.05/2.10	0.98
Ethylenediamine tetraacetic acid	CMC	125	2.13/1.69	1.26
α-Hydrazino-w-cyclohexyl butyric acid	CMC	60	1.31/1.73	0.76
Citrinin	CMC	20	3.27/3.43	0.95
Mercury hematoporphyrin complex sodium	S	10	3.72/2.86	1.30
Podophyllotoxin	A and CMC	2.0	1.30/1.63	0.80
Citral	PO	250	1.62/1.69	0.96
Zymosan	CMC	5.0	2.16/1.96	1.10
Zymosan	CMC	200	1.97/1.96	1.00
Potassium arsenite	S	8.0	1.53/1.63	0.94
H_2O_2		0.3% in drinking water	1.72/1.50	1.23
Carbon black	S	320**	2.66/2.65	1.00

* Spleen weight of treated animals/spleen weight of control leukemic animals.
** Given intravenously.

It is apparent from the results that 40 of 110 compounds tested had no inhibitory effect on Friend virus leukemia (Table 1). Colchicine, zymosan, 5-fluorodeoxyuridin, 3,3-diethyl-1-phenyltriazene, DON, podophyllotoxin, and H_2O_2 belonged to this ineffective group. It is interesting that many antibiotics also belonged to the negative group. Furthermore, we have tested many crude preparations of antibiotics and have found several which had a stimulating effect on the Friend virus leukemia. Six compounds also had a stimulating effect on the leukemia.

Table 2. Slightly Inhibitory Compounds on Friend Mouse Virus Leukemia

Compound	Solvent	Dose mg/kg/day	Av. spleen weight at 3 rd week (g) treated/controls	Tumor index
A-Methopterin	S	1.5	1.28/2.64	0.54
Sarkolysin	S	2.0	1.87/3.43	0.53
Purine	S	250	1.09/1.63	0.67
2, 6-Diaminopurine	S	60	0.93/1.69	0.55
8-Azaguanine	CMC	75	1.19/1.72	0.68
1-Methyl-3-nitro-1-nitrosoguanidine	CMC	10	1.23/1.69	0.73
1, 2-Di (methanesulfonoxy) ethane	CMC	100	1.76/2.64	0.67
Purine-6-thioglucoside	S	250	1.58/2.88	0.55
5-Fluorouridine	S	5.0	1.10/1.87	0.59
5-Fluorodeoxycytidine	CMC	50	1.33/2.53	0.52
5-Bromodeoxyuridine	S	500	1.40/1.91	0.73
Pentamycin	S	1.0	1.81/2.64	0.68
Actidione benzoylacetate	CMC	15	1.26/2.34	0.54
Actinobolin	S	1000	1.38/2.41	0.57
Streptovitacin A	S	0.3	1.50/2.41	0.62
Cortisone acetate	S	37.5	0.92/1.46	0.63
1-Dehydro-9α-fluoro-hydrocortisone acetate	CMC	12.5	1.30/1.81	0.72
3β-Methoxy-Δ^5-androsten-16β-ol	S	200	1.65/2.34	0.70
5-Chloro-2-galactobenzimidazole	CMC	150	0.88/1.47	0.60
p-Nitrobenzenediazoaminobenzene	CMC	500	1.10/1.87	0.59
Diaziridinyl phosphonitrile	S	0.25	1.96/2.64	0.74
Alazopeptin	S	0.1	1.42/1.94	0.73
Cinnamylidene-anhydroacetone benzil	CMC	250	2·32/3.43	0.63
3-Amino-4, 6-dihydroxy-α-isopropyl-amino-acetophenone·HCl	CMC	500	1.17/2.63	0.58
Milk feeding		*ad lib.*	1.29/1.94	0.66

A-Methopterin, cortisone, purine, 2, 6-diaminopurine, 5-fluorouridine, actinobolin, and sarkolysin were among 25 compounds that had only a slight inhibitory effect on the Friend virus leukemia (Table 2).

Twenty-eight compounds had a maderate inhibitory effect on Friend virus leukemia (Table 3). HN_2, triethylene thiophosphoramide, purine-6-thioglucoside, 5-fluorouracil, 5-fluoroorotic acid, diethylstilbestrol, *o*-phenylenediamine, 4-nitro-

quinoline-N-oxide, and azaserine belonged to this group. The difierence in the effects of DON and azaserine is considerable. This may be another reflection of the difference in pharmacology of these two antibiotics.

Nine out of 110 compounds had a marked inhibitory effect on Friend virus leukemia (Table 4). Urethane, *p*-phenylene bis(diethyleneimidophosphate), 6-mercaptopurine, 6-mercaptopurine riboside, and 3,3-dimethyl-1-phenyltriazene belonged to this effective group. Purine, 8-azaguanine, and 2,6-diaminopurine were ineffective against Friend virus leukemia, but 6-mercaptopurine, 6-mercaptopurine riboside, and 6-(2,2-dimethylhydrazino)-purine showed a great activity against

Table 3. Moderately Inhibitory Compounds on Friend Mouse Virus Leukemia

Compound	Solvent	Dose mg/kg/day	Av. spleen weight at 3rd week (g) treated/controls	Tumor index
HN_2	S	0.5	0.71/1.88	0.38
Triethylene thiophosphoramide	S	4.0	0.50/1.46	0.34
2,6-Diacetamidopurine	CMC	160	0.61/1.88	0.32
2-Hydrazinopurine	CMC	500	0.80/1.69	0.47
Purine-6-thioglucoside	S	250	1.00/2.22	0.45
2-Amino-6-(1′-methyl-4′-nitro-5′-imidazolyl)-mercaptopurine	CMC	5.0	1.08/3.43	0.31
Thioguanine riboside	CMC	5.0	0.60/1.93	0.31
Thymine	CMC	500	0.80/1.73	0.46
2,4-bis (Ethyleneimino)-6-chloro-pyrimidine	S	2.0	0.48/1.63	0.30
2,6-Diamino-3-phenylazopyridine·HCl	S	125	1.30/2.86	0.45
5-Fluorouracil	S	35	0.63/1.32	0.48
5-Fluoroorotic acid	S	200	0.80/2.88	0.28
3,3-Dimethyl-1-*p*-tolyl triazene	CMC	100	0.72/1.72	0.42
3,3-Dimethl-1-*p*-nitrophenyl triazene	CMC	250	0.51/1.18	0.43
3-Cyclohexyl-3-methyl-1-*p*-tolyl triazene	PO	250	0.56/1.63	0.34
3,5-Dihydroxy-1,2,4-triazine	CMC	500	0.71/1.93	0.37
2-Allylimino-1,3,4-thiadiazoline·HCl	S	100	0.59/1.40	0.42
8-Nitro-1-methyl-2-quinolone	CMC	125	0.48/1.63	0.30
Azaserine	S	5.0	0.69/1.46	0.47
Actinomycin C	A and S	0.05	0.33/1.25	5.26
Diethylstilbestrol	CMC	10	0.85/1.90	0.45
17-Ethinyl testosterone	CMC	375	0.66/1.49	0.44
17-Ethinyl 19-nortestosterone	CMC	500	0.52/1.47	0.35
17-Ethinyl-*L*-glutamine 19-nortestosterone	CMC	500	0.58/1.94	0.35
4-Nitroquinoline-N-oxide	CMC	10	0.55/1.69	0.32
6-Aminonicotinamide	CMC	5.0	0.67/1.69	0.40
p-[N,N-bis (2-Iodoethyl) amino]-benzylphosphoric acid, diethyl ester	CMC	15	0.54/2.03	0.27
o-Phenylenediamine	S	200	0.78/1.96	0.40

this virus leukemia. 3,3-Dimethyl-1-phenyltriazene had a distinct inhibitory effect on Friend virus leukemia, but its analog, 3,3-diethyl-1-phenyltriazene, had no effect.

Table 4. Markedly Inhibitory Compounds on Friend Mouse Virus Leukemia

Compound	Solvent	Dose mg/kg/day	Av. spleen weight at 3 rd week (g) treated/controls	Tumor index
Urethane	S	500	0.30/1.49	0.20
p-Phenylene bis(diethyleneimidophosphate)	S	40	0.20/1.18	0.17
6-Mercaptopurine	CMC	30	0.27/1.36	0.17
6-Mercaptopurine riboside	CMC	125	0.36/1.88	0.19
6-(1′-Methyl-4′-nitro-5′-imidazolyl)-mercaptopurine	CMC	100	0.54/2.32	0.23
6-(2, 2-Dimethylhydrazino)-purine	CMC	100	0.53/2.28	0.23
3, 3-Dimethyl-1-phenyltriazene	PO	50	0.43/1.77	0.24
p-(Dimethyltriazeno) benzensulfonamide	CMC	175	0.40/1.62	0.25
Purinyl-6-thiocyanate	CMC	15	0.44/1.94	0.23

Table 5. Completely Inhibitory Compounds on Friend Mouse Virus Leukemia

Compound	Solvent	Dose mg/kg/day	Av. spleen weight at 3 rd week (g) treated/controls	Tumor index
Triethylene melamine	S	0.5	0.22/2.42	0.09
Thioguanine	CMC	2.0	0.27/2.34	0.11
Myleran	PO	20	0.19/1.96	0.10
1, 9-di(Methanesulfonoxy)nonane	PO	50	0.20/2.34	0.08
Mitomycin C	S	2.0	0.16/2.34	0.07
9α-Fluoro-2α-methyl-hydrocortisone acetate	CMC	37.5	0.23/1.58	0.14
Estradiol	CMC	40	0.25/1.72	0.14
Netropsin·HCl	CMC	30	0.41/3.43	0.12

Eight compounds had a complete inhibitory effect on Friend virus leukemia (Table 5). These compounds are three alkylating agents, namely, triethylene melamine, and 1,9-di (methanesulfonoxy)-nonane, two antibiotics, mitomycin C and netropsin, one purine, thioguanine, and two steroids, 9-α-fluoro-2α-methyl-hydrocortisone acetate, and estradiol. 1,4-Di(methanesulfonoxy)butane and 1,9-di-(methanesulfonoxy)nonane were very effective on the leukemia, but the lower homolog, 1,2-di(methanesulfonoxy)ethane was almost inactive. Triethylene melamine had a complete inhibitory effect on Friend virus leukemia, but triethylene thiophosphoramide had a moderate inhibitory effect. With four good anti-leukemia compounds we made further studies on bioassay, titration of the leukemia virus in the treated and untreated leukemic spleens, and determination of increase in survival time of treated animals.

To test for the presence of the virus, 10 per cent spleen homogenates were prepared with two to three small spleens selected from seven to ten leukemic mice treated with compounds, and 0.2 cc of the homogenates was then injected intraperitoneally into normal mice. Examination of spleens of these mice at the end of 3 weeks after intraperitoneal injection of leukemic spleen homogenates from mice which had been previously treated with triethylene melamine (0.5 mg/kg/day), myleran (20 mg/kg/day), 1,9-di(methanesulfonoxy)nonane (50 mg/kg/day) and mitomycin C (1 to 2 mg/kg/day) showed normal-sized spleens or smaller, indicating the absence of leukemia. On the other hand, bioassay of spleens of leukemic mice treated with compounds such as colchicine, HN_2, purine, 5-fluorouracil, cortisone, azaserine, actinomycin D, and H_2O_2 gave very large spleens.

For more definitive evaluation of the results we titrated Friend leukemia virus in pooled spleens (7 to 12 pieces) of untreated and treated mice. The results shown in Table 6 are the average of 2 determinations using 10 animals for each dilution.

Table 6. Presence of Friend Mouse Leukemia Virus As Determined by Spleen Weight* after Chemotherapy**

Dilution	Average weight of spleens (g) in mice receiving various dilutions			
	Control	TEM 0.5 mg/kg/day	Nonane[1] 25 mg/kg/day	Mitomycin 2 mg/kg/day
10^{-1}	2.05	0.34	1.56	1.20
10^{-2}	1.55	0.26	0.50	0.40
10^{-3}	1.49	0.23	0.27	0.25
10^{-4}	0.35	0.20	0.21	0.23
10^{-5}	0.25	0.20	0.18	0.17
10^{-6}	0.21	0.18	0.20	0.22

* Three weeks after intraperitoneal inoculation of virus.

** Treatment started one day post inoculation.

[1] 1,9-Di (methanesulfonoxy) nonane.

It is evident from Table 6 that the 50 per cent end point in the control group is between 10^{-3} and 10^{-4}. In the case of the triethylene melamine treated group the 50 per cent end point is less than 10^{-1}, while for 1,9-di(methanesulfonoxy) nonane and mitomycin C it is close to 10^{-2}. These results show a significant difference.

In order to evaluate the chemotherapeutic activity of these four compounds by means of their ability to prolong the survival time of mice with transplanted virus leukemia the following experiments were undertaken. In a typical experiment approximately 200 female Swiss albino mice were injected intraperitoneally with 0.2 cc of a 10 per cent saline homogenate of leukemic spleens. This transplanted leukemia kills mice in 2 to 4 months with marked enlargement of liver and spleen. Twenty-four hours after inoculation these mice were divided into

comparable groups of 20 mice each and treated intraperitoneally once daily for 7 days. The animals were treated at maximum and half maximum tolerated doses. These animals were allowed to live 4 months and then sacrificed for examination of visceral organs. During this period it was found that compounds tested at the higher doses were toxic and many animals died long before the experimental period of 4 months. From 20 leukemic mice in each group treated with 0.5 mg/kg/day of triethylene melamine 8 mice survived 2 months; with 20 mg/kg/day of myleran 2 mice survived; with 50 mg/kg/day of nonane 2 mice surved, and with 2 mg/kg/day of mitomycin C 5 mice survived 2 months. Although most of the spleens and livers of shese animals were small, no definite conclusion can be made due to the short survival time. Therefore, we made an evaluation from the results obtained with half these doses.

Table 7. Survival of Mice Treated with Triethylene Melamine, Myleran, 1, 9-Di(methanesulfonoxy)nonane, and Mitomycin C After Inoculation with Friend Mouse Virus Leukemia*

Duration in months	Control	TEM 0.25 mg/kg/day	Myleran 10 mg/kg/day	Nonane 25 mg/kg/day	Mitomycin C 1 mg/kg/day
1	17	19	10	15	17
2	9	14	6	10	14
3	4	11	4	9	12
4	0	9	0	5	10

It is evident from the data in Table 7 that none of the control animals survived 4 months, whereas 50 per cent of the mice treated with 0.25 mg/kg of TEM, and 1 mg/kg of mitomycin C daily for 7 days survived 4 months. Of 9 spleens of mice treated with TEM 3 were large and 6 were small. Of 10 spleens of mice treated with mitomycin C 2 were medium size spleens and 8 were normal size spleens (Fig. 2). Myleran showed less anti-leukemic activity than the nonane analog. Of 5 spleens of mice treated with the nonane analog 1 was large and 4 were small, whereas none of the mice treated with myleran lived 4 months. The above experiments were repeated with mitomycin C, TEM, nonane, and myleran to obtain survival time of mice. Results were essentially the same as the previous findings.

In general mitomycin C showed more anti-leukemic activity than did the other three compounds. It may be of interest to mention here that mitomycin C, a new antibiotic isolated from *Streptomyces caespitosus* (2), had a strong antitumor action on 16 of 30 various tumors (3) as shown in Tables 8 and 9.

Histological examinations of liver, spleen and bone marrow of leukemic mice 3 months after intraperitoneal injection of the leukemic spleen homogenates revealed focal collection of leukemic cells in the liver (Fig. 3), loss of demarcation of the follicles and the red pulp and replacement by leukemic cells in the spleen (Fig. 5). The bone marrow showed many mononuclear cells. On the other hand, the liver,

Table 8. Effect of Mitomycin C on Mouse Tumors (Dose: 2 ug/kg/day)

Tumor	Inhibition
Sarcoma 180 (ascitic)	##
Ehrlich carcinoma (ascitic)	##
Carcinoma 1025	##
Ridgway osteogenic sarcoma	##
Friend virus leukemia	##
Adenocarcinoma E 0771	#
Miyono adenocarcinoma	#
Harding-Passey melanoma	#
Glioma 26	#
Sarcoma 180	+
Bashford carcinoma 63	+
Lewis lung carcinoma	+
Wagner osteogenic sarcoma	+
Leukemia L 1210	+
Sarcoma T 241	±
Sarcoma MA 387	±
Ehrlich carcinoma	±
Lewis bladder carcinoma	±
Mecca lymphosarcoma	−
Gardner lymphosarcoma	−
Leukemia L 4946	−

indicates complete inhibition; # indicates marked inhibition;
+ indicates moderate inhibition; ± indicates slight inhibition;
− indicates no effect.

Table 9. Effect of Mitomycin C on Rat and Hamster Tumors (Dose: 1 mg/kg/day)

Tumor	Inhibition
Flexner-Jobling carcinoma	##
Walker carcinosarcoma 256	##
Jensen sarcoma	##
Iglesias ovarian tumor	##
Murphy-Sturm lymphosarcoma	#
Iglesias adrenal tumor	#
Crabb hamster sarcoma	#
Fortner hamster pancreas carcinoma	−
Fortner hamster intestinal carcinoma	−

indicates complete inhibition; # indicates marked inhibition;
− indicates no effect.

spleen and bone marrow of leukemic mice treated with mitomycin C, triethylene melamine, 1,9-di(methanesulfonoxy)nonane, and myleran showed a picture similar to that of normal mice (Figs. 4, 6). There were no leukemic cells in the liver, spleen, or bone marrow.

Mitomycin C at levels of 1 to 2 mg/kg/day, triethylene melamine at 0.5 mg/kg/day, and 1,9-di(methanesulfonoxy)nonane at 50 mg/kg/day had a marked inhibitory effect on 7-day-old Friend virus leukemia and caused its complete destruction in about 50 per cent of cases as proven by bioassay (a total of 120 Swiss albino mice were used).

Discussion

Since the Friend mouse virus leukemia responded well to treatment with mitomycin C, we tested its antitumor activity on another virus tumor, Rous chicken sarcoma. Results showed that subcutaneous or intraperitoneal injections of 2 mg/kg/day of mitomycin C for 7 days had a marked inhibitory effect on the growth of Rous chicken sarcoma in young chickens, but 1 mg/kg/day had practically no effect. Although 3 mg/kg/day of mitomycin C completely inhibited tumor growth, it was found to be lethal for many of the hosts (Fig. 7). Daily maximum tolerated does of 0.5 mg/kg of triethylene melamine and 50 mg/kg of 1,9-di(methanesulfonoxy)nonane had only a slight inhibitory effect on Rous chicken sarcoma. Against Friend virus leukemia, however, these compounds showed a maximum inhibitory effect equal to that of mitomycin C.

In connection with the ***in vivo*** investigation, a study was made to test the possible direct antiviral action of mitomycin C ***in vitro***. Two-cc portions of the filtrate of a 20 per cent saline homogenate of leukemic spleens were treated with 100, 500, and 1000 γ/cc of mitomycin C and were incubated for 24 hours at 4° to 5°C. After incubation the mitomycin C was dialyzed out from cellulose tubing, and 0.2 cc of the mitomycin C-treated Friend virus leukemia solution was then injected into normal mice intraperitoneally. Results showed that 100 and 500 γ/cc of mitomycin C for 24 hours' incubation at 4° to 5°C had no inhibitory effect, whereas 1000 γ/cc had only a slight inhibitory effect on the subsequent development of leukemia. It is interesting to note that the transplantability of both Ehrlich ascites carcinoma and Sarcoma 180 ascites tumor, which are not virus tumors, was completely destroyed by exposure to 10 γ/cc and 1 γ/cc of mitomycin C, respectively, for 24 hours at 4° to 5°C.

Inspection of Tables 1 to 5 show that triethylene melamine (4), myleran (5), 1,9-di(methanesulfonoxy)nonane (6), mitomycin C (3), urethane (7,8), 6-mercaptopurine (9,10), thioguanine (7,8), and 3,3-dimethyl-1-phenyltriazene (11) have shown a marked inhibitory to destructive effect upon the Friend virus leukemia. These

same compounds also have a good antitumor action on many solid and ascites tumors. On the other hand, the strong antitumor compounds fumagillin (12), actinomycin D (12), 5-fluorodeoxyuridine (13), 6-diazo-5-oxo-*L*-norleucine (14, 21) on solid and ascites tumors were inactive against Friend virus leukemia. Similarly good antitumor compounds cortisone (7, 8), sarkolysin (15), and amethopterin (16, 17, 18, 19) had only a slight inhibitory effect on the Friend virus leukemia. HN_2 (20), 5-fluorouracil (22), azaserine (23), and 4-nitroquinoline-N-oxide (24) have shown a definite inhibitory effect upon the growth of both Friend virus leukemia and several solid and ascites tumors.

Friend mouse virus leukemia appears to have wide applicability as a tool in chemotherapy screening, not only because of its neoplastic nature, but also since it is of demonstrated viral origin. Compounds having an inhibitory action on the leukemia may have several modes of action—e.g., destruction of leukemic cells, inhibition of virus proliferation, and direct inactivation of the virus. Thus the use of this virus-induced leukemia adds to the amount of useful information gained by the screening of potential chemotherapeutic agents.

Summary

1. Effects of 110 selected compounds tested against the Friend mouse virus leukemia are reported.

2. Mitomycin C, triethylene melamine, 1, 9-di(methanesulfonoxy)-nonane, and myleran had a destructive effect on the Friend virus leukemia as seen in spleen weight, bioassay and increase in survival time.

3. Urethane, *p*-phenylene bis(diethyleneimido phosphate), 6-mercaptopurine, 6-mercaptopurine riboside, thioguanine, 6-(1'-methyl-4'-nitro-5'-imidazolyl)-mercaptopurine, 6-(2, 2-dimethylhydrazino)-purine, purinyl-6-thiocyanate, 9α-fluoro-2α-methylhydrocortisone acetate, estradiol, 3, 3-dimethyl-1-phenyltriazene, *p*-(diethyltriazeno) benzenesulfonamide, and netropsin had a markedly inhibitory effect on the Friend virus leukemia.

4. Of the better-known materials HN_2, triethlene thiophosphoramide, purine-6-thioglucoside, thioguanine riboside, 5-fluorouracil, thymine, diethylstilbestrol, azaserine, actinomycin C, 8-nitro-1-methyl-2-quinolone, 4-nitroquinoline-N-oxide, and 6-aminonicotinamide had a moderately inhibitory effect on the Friend virus leukemia, and A-methopterin, 2, 6-diaminopurine, 8-azaguanine, 5-fluorouridine, 5-fluorodeoyuridine, colchicine, cortisone, sarkolysin, chloramphenicol, 6-diazo-5-oxo-*L*-norleucine, fumagillin, actinomycin D, podophyllotoxin, ethylenediamine tetraacetic acid, zymosan, potassium arsenite, and H_2O_2 had practically no inhibitoy effect.

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Legends for Platees XVI~XVIII

Fig. 1. The mouse on the left was killed 8 weeks after intraperitoneal injection of leukemic spleen homogenate. There is marked enlargement of liver and spleen (50 g). The mouse on the right in an uninjected control. The spleen weighed 0.18 g.

Fig. 2. The top row illustrates the spleens of leukemic mice 3 months after intraperitoneol injection of leukemic spleen homogenate. (Spleens weighed from 2.5 to 5.0 gm). The bottom row illustrates spleens of leukemic mice 3 months after treatment with mitomycin C at daily doses of 1 mg/kg on seven consecutive day. (Spleens weighed from 0.12 to 0.5 g).

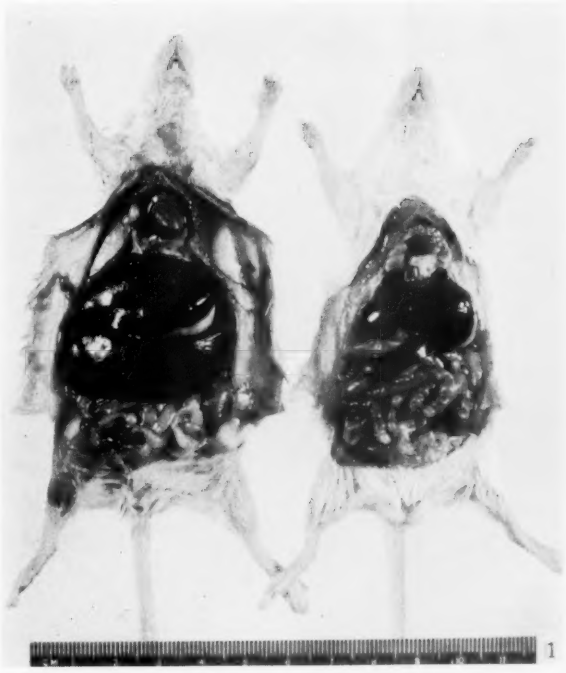
Fig. 3. Portal infiltration by leukemic cells in the liver of a mouse sacrificed 3 months after intraperitoneal injection of the leukemic spleen homogenate. X 300.

Fig. 4. Section of the liver of a mouse sacrificed 3 months after taeatment with mitomycin C (1 mg/kg/day for seven days). It shows a picture of a normal liver. There are no leukemic cells in the liver. X 300.

Fig. 5. Spleen from the same untreated leukemic mouse (Fig. 3) 3 months after inoculation of virus leukemia showing a loss of demarcation of the follicles and the red pulp and replacement by leukemic cells. X 300.

Fig. 6. Section of the spleen of the same leukemic mouse (Fig. 4) treated with mitomycin C. It shows the histology of a normal spleen including presence of megakaryocytes. There are no leukemic cells in the spleen. X 300.

Fig. 7. The control chicken on the left shows a three-week-old Rous chicken sarcoma. The chicken on the right is a bird treated with mitomycin C (daily doses of 3 mg/kg of mitomycin C on seven consecutive days), showing complete destruction of the tumor.



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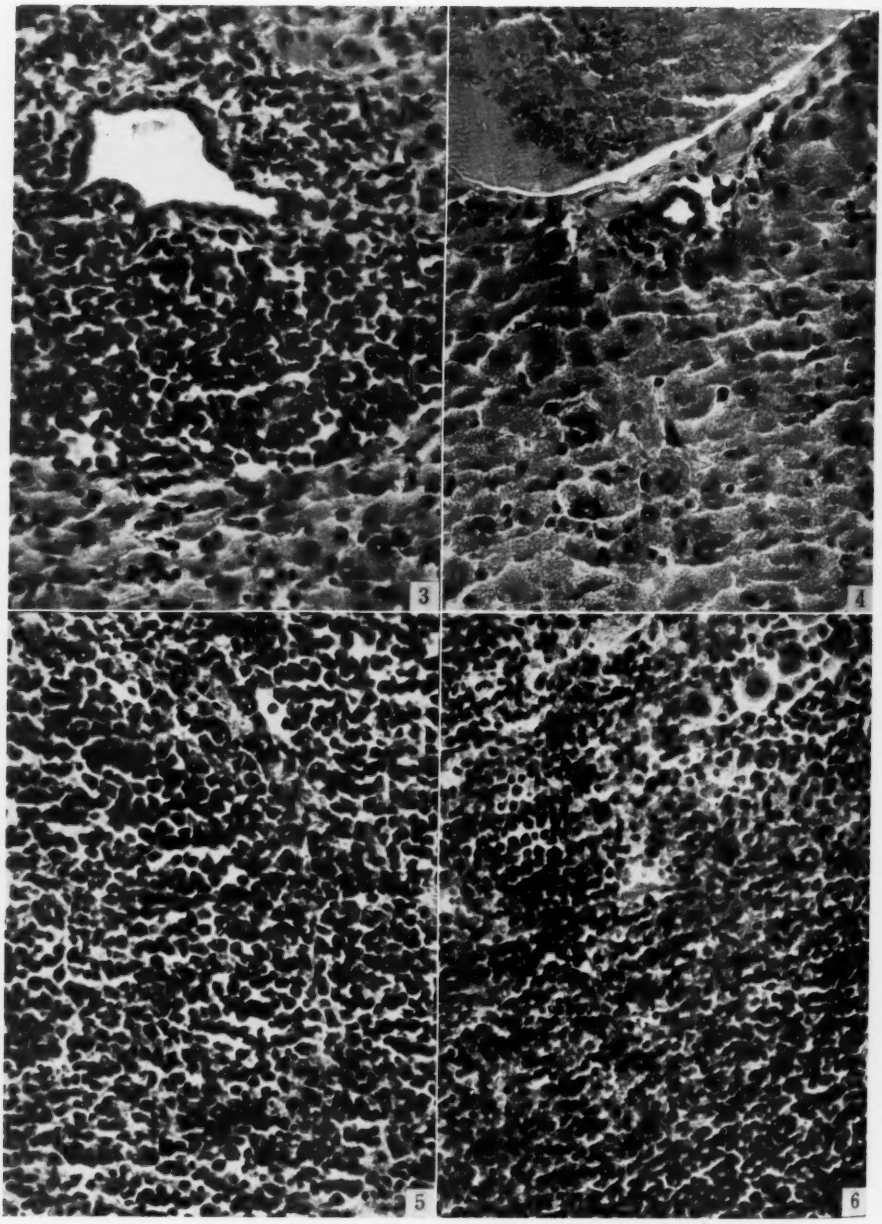
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[GANN, 50, 265~276; September, 1959]

STUDIES ON ANTI-TUMOR ACTIVITIES OF OXIMES AND METAL CHELATE COMPOUNDS

I. EXAMINATIONS OF OXIMES[1]

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INTRODUCTION

Hydroxylamine, a well known inhibitor of tissue catalase, was reported to show some antitumor activities for human skin-carcinoma[2], mouse ascites tumor cells[3], and other tumors[4]. The hydroxylamine yields oximes in the presence of carbonyl compounds, and the isonitroso group in the resulting oxime is transported to other carbonyl compounds. The oxime has been supposed to be an intermediate involved in one course of nitrogen metabolic systems[5].

The oxime may show the antitumor activity, as it carries the active hydroxylamine as its isonitroso group. Only a few of the antitumor activity of oximes have been examined and found to be non-effective on the tumor growth[6].

As the author has found that levulinic acid oxime exhibited the selective biological effects, such as the inhibitory action against Mycobacteria[7], he has also examined the antitumor activities of the acid oxime and others on Ehrlich carcinoma and Crocker sarcoma 180.

The possibilities to increase the antitumor activity of oximes in the presence of metal ions are also discussed in this paper.

EXPERIMENTAL

In vivo test: Ehrlich carcinoma and Crocker sarcoma 180 strains were obtained by courtesy of the Sixth Laboratory, Institute of Applied Microbiology, University of Tokyo, and were transplanted intraperitoneally into mice. All the tests were carried out with mice, five mice in a group, 15–20 g body weights, 4–5 weeks after their birth. The cell suspension in physiological saline (0.2 m*l*) containing a million cells of Ehrlich carcinoma or two million cells of sarcoma 180 was inoculated intraperitoneally into a mouse. Usually, during successive seven days starting one hour after the inoculation, the solutions containing a certain amount of compounds were injected into the peritoneal cavity, once a day. The antitumor activity was checked by body weight curves, survival periods, results of autopsy, etc..

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Water-soluble compounds were dissolved in the physiological saline and neutralized with sodium hydroxide to keep them at pH 7. The water-insoluble compounds were suspended in the saline containing 0.5% carboxylmethyl-cellulose (CMC) with the aid of a glass homogenizer. The control mice were injected the physiological saline, containing CMC or not, during the same days with the above mentioned experiments. The commercial ration, "Oriental compressed diet (MN) for experimental animals", and the physiological saline were supplied to the mice.

The in vitro contact tests: a) Stained cell count method[8)9)]. (The phosphate buffer was prepared by dissolving NaCl (16 g), $NaHPO_4$ (5.8 g), KCl (0.4 g), and KH_2PO_4 (0.4 g) in 1 *l* of water.)

The cell suspension of mouse Ehrlich carcinoma in the phosphate buffer (pH 7) was adjusted to contain 2×10^7 cells in each ml. Levulinic acid oxime (sodium salt) or other antitumor substances, such as nitromin or sarkomycin, was dissolved in one ml aliquot of the suspension. After incubating at 37°C, one part of the suspension was mixed with that of eosin solution (0.5% in physiological saline) on the slide. The percentage of cells stained with eosin was counted microscopically after standing 2 minutes.

Usually, the normal cells may not be stained with eosin, as their cell membranes have the so-called selective permeability. Once the cell membranes are injured by the administration of some substances to break the selective permeability, the cell may be stained with the eosin, as observed in the dead cells.

b) T.T.C. (2, 3, 5-triphenyl tetrazolium chloride) standing method[9)]. The solution of levulinic acid oxime or α-ketoglutaric acid oxime was neutralized with sodium hydroxide to pH 7, and diluted with the phosphate buffer. One ml aliquot of the sample solution was mixed with 1 m*l* of the cell suspension (4×10^7 Ehrlich carcinoma cells per m*l* of the buffer) with the use of a Tunberg tube. After standing 1 hour at 37°C, 1 m*l* of the T.T.C. solution (0.2% in the physiological saline) and 1 m*l* of the methylene blue solution (0.005% in the saline) were added to the sample solution. After standing one hour at the same temperature, the formed formazan was extracted with 4 ml of ethyl-acetate, and measured colorimetrically at 482 mμ, using Beckman model DU spectrophotometer. The amount of the formed formazan was decreased when the dehydrogenase activity of tumor cells was inhibited with oximes.

Formation of chelate compounds: The possibility of forming metal chelate compounds of oximes in the aqueous solution at pH 7 was examined by the ordinary titration method with sodium hydroxide (N/10).

Results

(1) *Levulinic acid oxime:* i) Against Ehrlich carcinoma in Cylinder Plate Me-

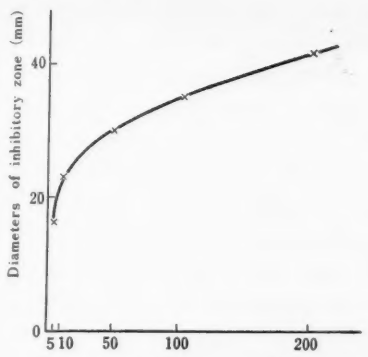

Fig. 1-a. Cylinder Plate Method; Inhibitory diameters of levulinic acid oxime at various concentrations on Ehrlich carcinoma.

thod of Testing Anticell Effects[10]. The inhibitory zone was clearly observed with levulinic acid oxime (Fig. 1-a).

ii) ***In vivo*** tests on Ehrlich ascites carcinoma and Crocker sarcoma 180: As shown in Figs. 1-b and 1-c, levulinic acid oxime, when administered daily for 7 or 12 consecutive days with doses of 1000, or 250 mg/kg/day, suppressed the body weight increase caused by the increase of ascites fluides.

iii) Acute toxicity: Levulinic acid oxime was administered intravenously to six groups, each consisting of five young mice weighing 16–18 g, in the dose of 650, 900, 1200, 1500, 1800, and 2100 mg/kg. The mortality was 0/5, 0/5, 0/5, 0/5, 1/5, and 5/5, respectively, after nine days observation. During these nine days animals kept their normal weights, except in the dose of 1500 and 1800 mg/kg which took somewhat longer days to gain their normal weights.

LD_{50}=2040 mg/kg (mice, intravenously)

(2) ***Stained cell count method*** (1): As shown in Fig. 2, levulinic acid oxime required a lesser amount (20 mg/m*l*) than α-ketoglutaric acid oxime (50–100 mg/m*l*) to cause the increase of the number of stained cells of Ehrlich carcinoma with

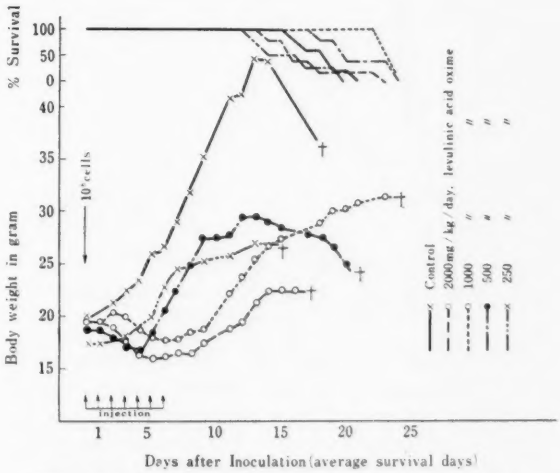

Fig. 1-b. ***In vivo*** test (1) (Ehrlich ascites carcinoma)

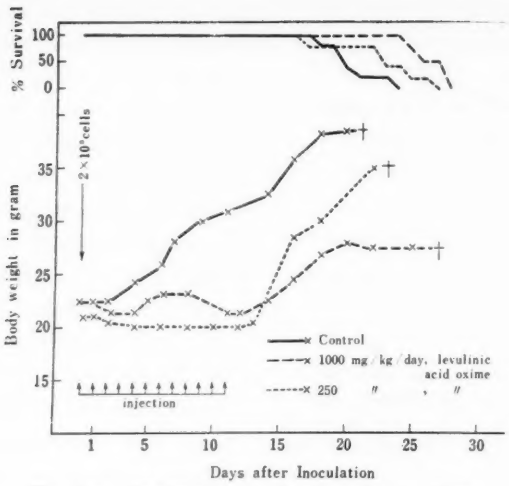

Fig. 1-c. *In vivo* test (2) (Crocker sarcoma 180)

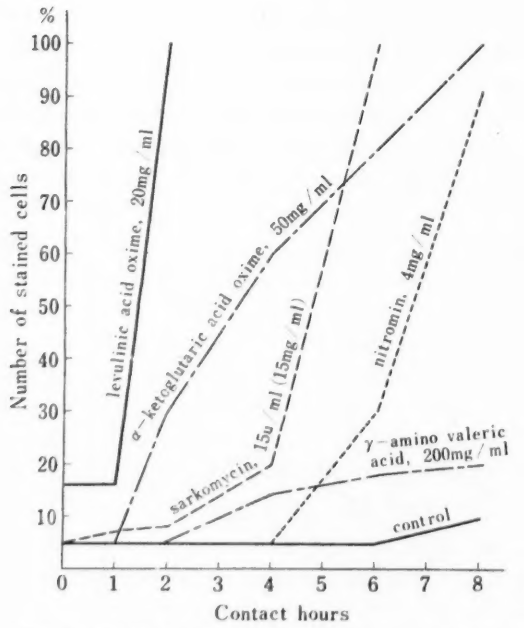

Fig. 2. Stained cell count method (1); The injurious effects of levulinc acid oxime and other antitmor substances on the cell-membranes of Ehrlich carcinoma.

eosin, showing their injurious effects on the cell membranes.

γ-Amino-valeric acid having γ-amino group instead of the γ-isonitroso group of levulinic acid oxime did not cause the injurious effect even at a concentration of 200 mg/m*l*. In the same experiment, nitromin and sarkomycin showed the same destructive action on the selective permeability of tumor cells at a concentration of 5 or 15 mg/m*l*, respectively.

(3) ***T.T.C. standing method:*** The activity of dehydrogenase in Ehrlich carcinoma was inhibited at a concentration of ca. 5 mg/m*l* by both levulinic acid oxime and α-ketoglutaric acid oxime. But, the γ-aminovaleric acid did not exhibit the clear inhibition even at concentrations of 50–100 mg/m*l*. These are shown in Table 1.

Table 1. T. T. C. Standing method; The inhibitory action of levulinic acid oxime, γ-amino-valeric acid, and α-ketoglutaric acid oxime on the dehydrogenase activity of Ehrlich carcinoma cells.

Compounds		T%
Levulinic acid oxime	10 mg/ml	95
	5 〃	91
	1 〃	38
	0.5 〃	28
	0.1 〃	7
α-Ketoglutaric acid oxime	10 〃	96
	1 〃	18
	0.5 〃	17
	0.1 〃	11
γ-Aminovaleric acid	250 〃	91
	100 〃	74
	50 〃	32
	10 〃	28
Control		11

(4) ***Levulinic acid derivatives:*** Levulinic acid having γ-keto group instead of the isonitroso group of levulinic acid oxime did not show the antitumor activity, showing no suppression of body weight increase due to the increase of ascites fluides and no prolongation of the survival periods, in the dose of 1000 mg/kg/day on Ehrlich carcinoma, and 1250 mg/kg/day on sarcoma 180. γ-Amino-valeric acid (450 mg/kg/day) and others were also found to be non-effective on the tumor growth at the ***in vivo*** test. These are shown in Table 2.

(5) ***Compounds having structural relationships with levulinic acid oxime, and others:*** As shown in Table 2, about 20 compounds were tested for their antitumor activities. These were compounds having structural relationships with levulinic acid oxime, compounds having adjacent terminal methyl-group to an isonitroso group, and general monoximes, such as gugar oximes. All of them did not show the

Table 2. *In vivo* tests on Ehrlich ascites carcinoma (E. C.) and Crocker sarcoma 180 (S-180).

Substances	Daily dose (mg/kg)	Tumor	No. of injection	Average survival days	Average survival time ratio, $\frac{treated}{controls} \times 100$ (%)	Suppression of body wt. increase
Hydroxylamine (free)	36	E. C.	7	19	126	(−)
Levulinic acid oxime	1000	E. C.	12	24	126	(+)
	500	E. C.	12	22	116	(+)
	1000	S-180	12	28	127	(+)
	250	S-180	12	24	110	(+)
Levulinic acid	1000	E. C.	7	20	100	(−)
	1000	S-180	7	24	90	(−)
γ-Aminovaleric acid	450	E. C.	7	22	100	(−)
	450	S-180	7	20	85	(−)
δ-Benzal-levulinic acid	100	E. C.	7	25	100	(−)
	100	S-180	7	18	90	(−)
2-Aminothiazolyl-4-propionic acid	1000	E. C.	7	15	90	(−)
2-Amino-4-methylthiazolyl-5-acetic acid	375	E. C.	7	15	100	(−)
α-Ketoglutaric acid oxime	614	E. C.	7	19	100	(−)
Dilevulinic acid dioxime	500	E. C.	7	19	100	(−)
Acetonyl acetone dioxime	500	E. C.	7	20	110	(−)
Acetaldoxime	88	E. C.	7	25	114	(−)
	80	S-180	7	20	90	(−)
Acetoxime	330	E. C.	7	28	130	(−)
	330	S-180	7	20	83	(−)
Methylethylketone oxime	170	E. C.	7	21	100	(−)
	170	S-180	7	18	75	(−)
Pyruvic acid oxime	160	E. C.	7	21	100	(−)
Acetoacetic acid oxime	380	E. C.	7	18	100	(−)
Benzaldoxime	216	E. C.	7	23	115	(−)
Acetophenoxime	410	E. C.	7	19	120	(−)
Cyclohexanone oxime	216	E. C.	7	20	100	(−)
	216	S-180	7	20	100	(−)
Mannose oxime	500	E. C.	7	18	100	(−)
Xylose oxime	500	E. C.	7	22	110	(−)

antitumor activity at the *in vivo* test.

(6) ***Stained cell count method*** (2), ***metal chelates of α-ketoglutaric acid oxime:*** As shown in Table 3, α-ketoglutaric acid oxime was found to show an immediate injurious action on the cell membranes of Ehrlich carcinoma at the concentration of over 100 mg/m*l*. This oxime was also found to be a chelating agent forming water-soluble chelates with copper and iron, as shown in Figs. 3 and 4. The

Table 3. Stained cell count method (2); The injurious effects of α-ketoglutaric acid oxime in the forms of its metal chelate compounds on the cell membranes of Ehrlich carcinoma.

Compounds \ Contact hours		Number of cells stained with eosin (%).					
		0	1	2	4	6	8
α-Ketoglutaric acid oxime[1]	100 mg/ml	17	40	100			
	50 〃	16	24	54	100		
	25 〃	17	20	76	90	100	
	10 〃	20	20	20	30	55	70
Nickel-α-Ketoglutaric acid oxime	× 1[2]	17	22	80	100		
	× 2		12	30	80	100	
	× 5		10	13	16	30	56
Copper-α-Ketoglutaric acid oxime	× 1[3]	100					
	× 2	100					
	× 5	50		50	50	54	56
Iron-α-Ketoglutaric acid oxime	× 1[4]	100					
	× 2	100					
	× 5	15	20		20		30
Control (P-buffer)		10	10		16		26

1) Dissolved into phosphate beffer (pH 7), and was neutralized with sodium hydroxide to pH 7.

2) α-Ketoglutaric acid oxime (50 mg, 2 M) and nickel nitrate (45 mg, 1 M) dissolved into the P-buffer (1 m*l*) and neutralized to pH 7.

3) α-Ketoglutaric acid oxime (50 mg, 2 M) and cupric sulphate (39 mg, 1 M) used.

4) α-Ketoglutaric acid oxime (50 mg) and ferrous sulphate (43 mg) dissolved into the buffer (1 m*l*) and neutralized with sodium hydroxide to pH 7.

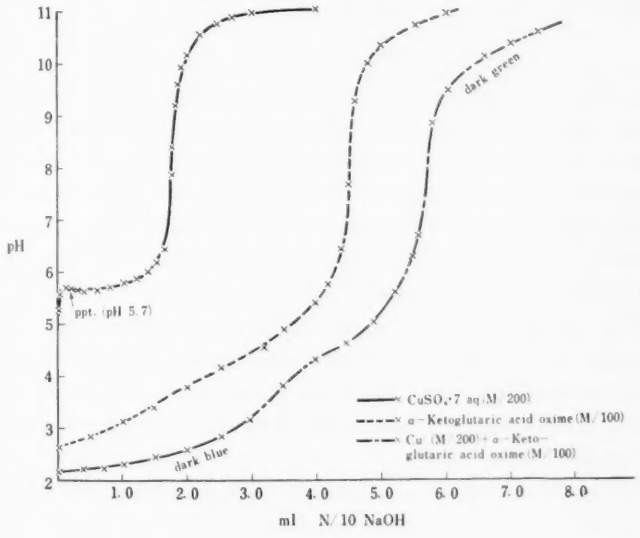

Fig. 3. The non-additive effect in titration, when complex formatiop takes place.

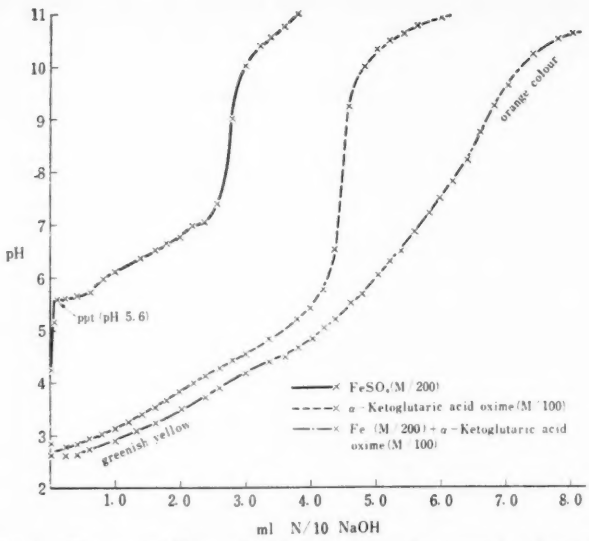

Fig. 4. The non-additive effect in titration, when complex formation takes place.

chelates were prepared by mixing the oxime (2M) and a metal ion (1M) at pH 7, as described in Table 3. In the cases of its copper and iron chelates, the immediate injurious actions were observed at a concentration of 25 mg/m*l* calculating as α-ketoglutaric acid oxime. The increased deterious effect was not observed with its nickel chelates.

Discussion

The reaction of hydroxylamine and levulinic acid yields levulinic acid oxime. The amine was already indicated to show the wide antimicrobial spectrum, and the oxime was reported to show a selective inhibitory action against Mycobacteria[7]. The hydroxylamine was also reported to exhibit the antitumor activity on mouse ascites tumor cells[3], skin-carcinoma[2], and Ehrlich adenocarcinoma[4]. It was supposed that levulinic acid oxime, carrying the active hydroxylamine as its isonitroso group might show some inhibitory actions against some carcinoma. (The hydroxylamine was reported to be noneffective on the growth of Crocker sarcoma 180 and Walker's adenocarcinoma[4]. In my experiments, it did not exhibit the activity in the dose of 36 mg/kg/day on Ehrlich ascites carcinoma. The reason why the amine did not show the clear antitumor activities ***in vivo*** might be due to the unstable nature of the hydroxylamine ***in vivo***[11].

The examination of the antitumor activity of levulinic acid oxime was performed to show that the oxime inhibited the growth of some tumors, both at ***in vivo*** and

in vitro tests. As shown in Fig. 2, at the *in vivo* test, the necessary concentration of levulinic acid oxime (20 mg/m*l*) to destroy the selective permeability of the cell membrane of Ehrlich carcinoma was about five times as that of nitromin (>4 mg/m*l*), and about the same with that of sarkomycin (>15 mg/m*l*). The toxicity of the levulinic acid oxime was low. The LD_{50} was 2040 mg/kg (mice, intravenously).

γ-Amino-valeric acid, having γ-amino group instead of the γ-isonitroso group of the oxime, did not show the injurious action on membranes of tumor cells even at the concentration of 200 mg/m*l* in the same stained cell count method. Levulinic acid oxime inhibited the dehydrogenase activity of tumor cells at the concentration of 5 mg/m*l*. But the γ-amino acid did not show the clear inhibitory action at concentrations of 50–100 mg/m*l* (Table 1). Moreover, the facts that the γ-amino-valeric acid or the levulinic acid, having γ-keto group, did not show the antitumor activity at the *in vivo* test (Table 2) might suggest the necessity of an isonitroso group of levulinic acid oxime to exhibit the antitumor activity.

The other oximes, having structural relationships with the levulinic acid oxime, might be expected to exhibit the antitumor activity, as they carried the active isonitroso group, but experiments failed to confirm it (Table 2).

For the antimicrobial activity[7], the existence of γ-isonitroso group of the levulinic acid oxime was indicated to be necessary, and other γ-isonitroso compounds, such as dilevulinic acid dioxime and acetonylacetone dioxime, also inhibited the growth of Mycobacteria. These dioximes did not show the antitumor activities (Table 2). Some other compounds having structural relationships did not afford the activity either.

α-Ketoglutaric acid oxime did not suppress the increase of ascites fluides in the dose of 614 mg/kg/day *in vivo*, but it showed thed estructive action on cell membranes of the carcinoma at concentrations of 50–100 mg/m*l*, and inhibited the dehydrogenase activity of tumor cells at a concentration of 5 mg/m*l*. The necessary dose to exhibit the injurious action on cell membranes of tumor cells differs between levulinic acid oxime (20 mg/m*l*) and α-ketoglutaric acid oxime, but they inhibited the dehydrogenase activity of the carcinoma at the same concentration (5 mg/m*l*). The difference may be concerned with the existence of the terminal carboxyl group (hydrophilic) in α-ketoglutaric acid oxime, instead of the terminal methyl group (lipophilic) of levulinic acid oxime. The hydrophilic group, such as carboxyl of α-ketoglutaric acid oxime, may hinder the easy permeation of the oxime through cell membranes of tumors consisting of the lipoid layer[12]. The hydrophilic group shall be masked to exhibit the antitumor activity.

Now, the chelating agent has been supposed to exert some biological activities robbing cells of metals, which are necessary for them to grow. Some chelating

agents, such as *o*-phenanthroline or α, α-dipyridyl, were already reported to show the antitumor activity on sarcoma 37 in the dose (30 mg/kg or 200 mg/kg, respectively) near the maximum tolerated (80–100 mg/kg or 250 mg/kg, respectively)[13].

α-Ketoglutaric acid oxime was found to be a strong chelating agent (Figs. 3 and 4), forming water-soluble chelates with copper, nickel, and iron at the physiological pH, but it did not inhibit the tumor growth in the doses of 500–600 mg/kg/day *in vivo* (Table 2).

If the chelating agent, α-ketoglutaric acid oxime, be introduced into tumor cells in the form of more permeable molecule against the cell membrane, it might inhibit the enzyme systems in the cells. For this purpose, the hydrophilic groups of the chelating agent, such as carboxyl groups and a nitrogen atom of the α-ketoglutaric acid oxime, should be masked in some way to be easily permeated into cells in the form of more non-ionic molecules. At the physiological pH, the hydrophilic group of oximes can be masked with co-existing metal ions, forming chelates compounds. During the permeation in tumor cells, the metal ion of the chelates may be robbed by other naturally occurring agents, such as amino acid residues

Table 4. Stained cell count method (3); The injurious effects of inorganic metals on the selective permeability of Ehrlich carcinoma cells.

Compounds	Contact hours	Number of cells stained with eosin (%)					
		0	1	2	4	6	8
Ni^{++}	× 1[1]	20	30	33	40	40	40
	× 2	23	23	25	30	35	35
	× 5	16	20	30	30	30	
	× 10		16	18	20	21	
Cu^{++}	× 1[2]	70	100				
	× 2	30	40	90	100		
	× 4	25	46	90	100		
	× 10	20	20	30	32	50	
Fe^{++}	× 1/2[3]	23	*				
	× 1	26	30	80	*		
	× 2	20	23	25	100*		
	× 5	16	16	16	16	100*	
Control		13	17	18	20	22	23

1) Nickel nitrate (11.2 mg) dissolved in water, and filled up to 1 ml after the neutralization with sodium hydroxide.

2) Cupric sulphate (39 mg) neutralized with sodium hydroxide to pH 7, and filled up to 1 ml.

3) Ferrous sulphate (86 mg) neutralized in the same way.

* A black viscous precipitate was deposited.

In the case of nickel, the physiological saline was applied to keep the metal water soluble. In the experiments with copper and iron, the phosphate-buffer (pH 7) was applied to cause the deposition of water insoluble metals.

of proteins. Then, the original chelating agent may show the inhibitory actions in tumor cells, robbing cells of their necessary metals to survive.

The immediate injurious action, on the selective permeability of cell membranes (Ehrlich carcinoma), was caused by α-ketoglutaric acid oxime at the concentration of over 100 mg/m*l*. The somewhat increased injurious action on tumor cells was observed with α-ketoglutaric acid oxime (25 mg/m*l*) in the forms of its copper and iron chelates, and not in that of nickel chelates (Table 3). The inorganic metals themselves did not show the destructive action on cell membranes at the same concentration with those were applied in the above mentioned experiments (Table 4). In this case, the metal ions masked the hydrophilic groups of the oxime, a carboxyl group and a basic nitrogen atom, to be more permeable through cell membranes. But the metal chelates of the α-ketoglutaric acid oxime might not be fully non-ionic, as a whole, as it carried the other non-reacted carboxyl group. If the more non-ionic and permeable molecules be prepared from oximes by forming chelates, the better antitumor activity may be observed.

Thus, dimethylglyoxime was found to exert a clear antitumor activity in the form of its copper chelate, as to be reported in my following papers.

Summary

The antitumor activities of oximes were examined on Ehrlich ascites carcinoma and Crocker sarcoma 180. 1) Levulinic acid oxime showed a clear antitumor activity at the ***in vitro*** test, and a slight inhibitory effect ***in vivo***. This oxime was found to show the destructive actions on cell membranes of the carcinoma at the concentration of 20 mg/m*l*, and to inhibit the activity of dehydrogenase at the concentration of 5 mg/m*l*. The necessary dose of the oxime to destroy the selective permeability of the cell membranes was about five times that of nitromin, and about the same with that of sarkomycin. The LD_{50} of the oxime was 2040 mg/kg (mice, intravenously). 2) The fact that γ-aminovaleric acid and levulinic acid did not show the activity suggested the necessity of the isonitroso group of levulinic acid oxime for the display of the activity. 3) About 20 monoximes and others having structural relationships with levulinic acid oxime were examined for their activities, but none showed the inhibitory actions on tne tumors ***in vivo***. 4) α-Ketoglutaric acid oxime was found to be a chelating agent and to form chelates with copper, iron, and nickel at the physiological pH. The injurious action of the oxime on the selective permeability of cell membranes of the tumor was increased in the presence of copper and iron in the forms of its chelate compounds. The metal ions might mask the hydrophillic groups of the oxime to be permeable through cell membranes, and to increase the antitumor activity.

The author wishes to express his sincerest thanks to Prof. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo, for his encouragement and guidance throughout this experiment. The author is very thankful to Prof. H. Umezawa for his kind encouragement and to Dr. H. Yonehara, Dr. N. Tanaka, and all other members of Sixth Laboratory, Institute of Applied Microbiology, University of Tokyo, for their encouragement and aid. He is indebted to Dr. M. Miyazawa and Dr. H. Kinukawa, Ōkubo Hospital, Tokyo, for tests by the cylinder plate method. The author is also thankful to Ajinomoto Co., L., for their contributions to this experiment, and to the Ministry of Education, Japanese Government, for a grant in aid.

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STUDIES ON ANTI-TUMOR ACTIVITIES OF OXIMES AND METAL CHELATE COMPOUNDS

II. COPPER-DIMETHYLGLYOXIME[1]

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Introduction

The chelating agent has been supposed to exert some biological activities by robbing cells of the necessary metals for them to survive[2]. O-Phenanthroline and bipyridyl, well known as chelating agents, were reported to show the antitumor activity on sarcoma 37, but many O-phenanthrenes and biphenyls did not exhibit the activity within their tolerable doses[3]. If the chelating agent be introduced into tumor cells in the form more permeable to the cell membranes, it may better inhibit enzyme systems in tumor cells, robbing them of metals.

As described in my previous paper[4], the destructive action of α-ketoglutaric acid oxime on the selective permeability of tumor cells was increased by forming its copper and iron chelates. The oxime was already indicated to be a chelating agent[4]. In this case, the metal ions might mask the hydrophilic groups of the α-ketoglutaric acid oxime, to be more permeable through cell membranes of Ehrlich carcinoma, and to increase the antitumor activity, as the membranes were indicated to consist of the lipoid layer[5]. But, the chelate compound was not fully non-charged molecules, as it carries the other non-reacted hydrophilic carboxyl group of α-ketoglutaric acid oxime. If the more non-ionic molecules be prepared from the chelating agent, the better antitumor activity may be observed.

Chelating with metals, dimethylglyoxime, a chelating agent, was assumed to form the stable, non-ionic molecule to be easily permeated in tumor cells. In this paper, antitumor activities of copper-dimethylglyoxime are presented.

Experiments and Results

The same methods for examination of antitumor activities as described in the previous paper[4] was applied in this experiment, both in ***in vivo*** and ***in vitro*** tests. Ehrlich carcinoma and Crocker sarcoma 180 were put to use, both in ascites and solid forms. The intraperitoneal injection of each compound was initiated 2 or 24 hours after the tumor inoculation.

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1) ***The antitumor examination of dimethylglyoxime.*** Dimethylglyoxime did not exhibit the antitumor activities on Ehrlich ascites carcinoma and sarcoma 180, even in the dose of 50 mg/kg/day. The oxime was injected intraperitoneally into mice during consecutive 7 days, once a day, starting one hour after the tumor inoculation. The sample solution was prepared suspending the dioxime into the physiological saline containing CMC (0.5%), as the oxime was hardly soluble in water.

2) ***The antitumor activity of dimethylglyoxime in the presence of metal ions.*** The

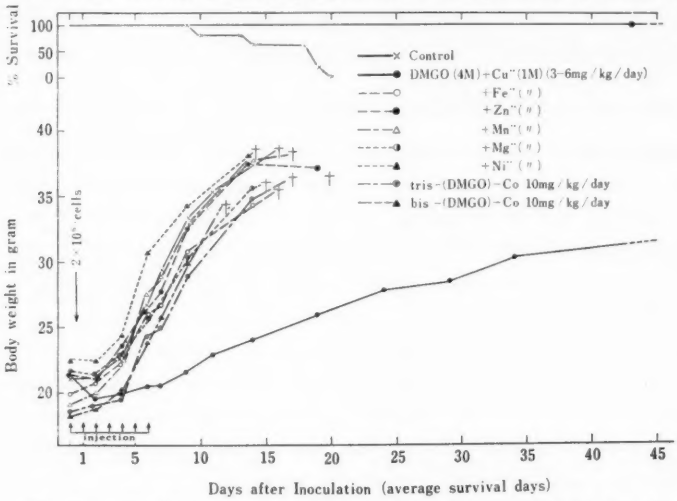


Fig. 1-a. *In vivo* test (1), Antitumor activities of dimethylglyoxime in the presence of metal ions (on sarcoma 180).

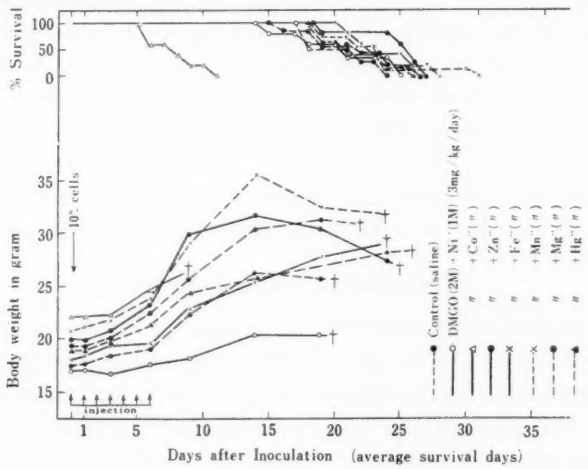


Fig. 1-b. *In vivo* test (2), Antitumor activities of dimethylglyoxime in the presence of metal ions (on Ehrlich carcinoma).

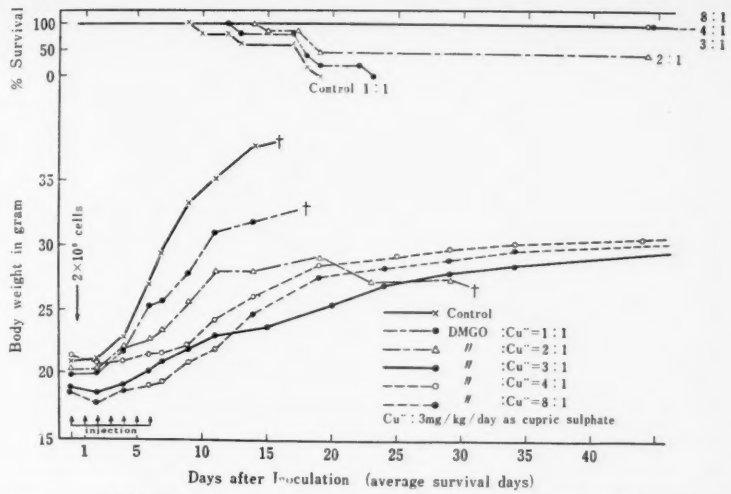
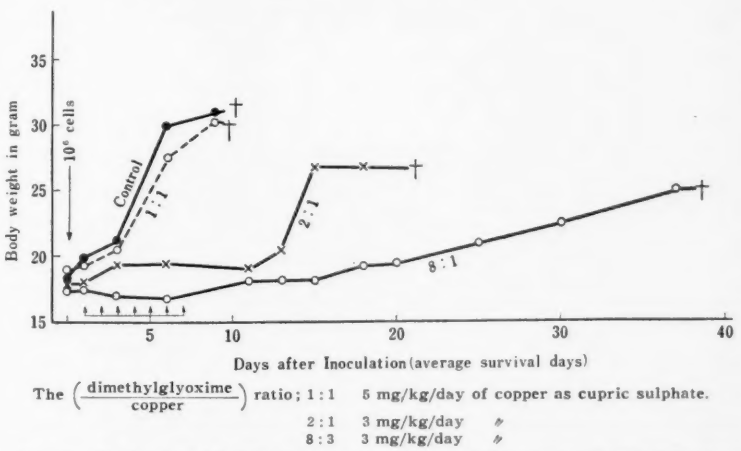


Fig. 2. *In vivo* test (3), The antitumor activity of the copper-dimethylglyoxime on ascites form of sarcoma 180.



The $\left(\frac{\text{dimethylglyoxime}}{\text{copper}}\right)$ ratio; 1 : 1 5 mg/kg/day of copper as cupric sulphate.
2 : 1 3 mg/kg/day 〃
8 : 3 3 mg/kg/day 〃

Fig. 3. *In vivo* test (4), The antitumor activity of the copper-dimethylglyoxime on ascites form of Ehrlich carcinoma.

Figs. 1-a and 1-b show that dimethylglyoxime (3-6 mg/kg/day) do not inhibit the tumor growth in the presence of metal ions (1/2 or 1/4 moles of the oxime), such as nickel, cobalt, zinc, iron, manganese,magnesium, and mercury. The oxime exhibited the antitumor activity only in the presence of copper ion in the dose of 3-6 mg/kg/day (Figs. 1-a, 2, and 3). Dimethylglyxoxime (10 mg/kg/day) was treated with 5 mg/kg of mercury nitrate (M/4 of the dioxime) at pH 7. But this test solution did not exhibit the antitumor activity. Each test solution was prepared ad-

mixing dimethylglyoxime (5 mg/kg) and a metal ion (M/2 or M/4 of the oxime). After standing a day, each solution was neutralized with sodium hydroxide to pH 7. Sulphate of copper, cobalt, zinc, iron (ferrous sulphate), and magnesium, nitrate of nickel, and manganese chloride were put to use, respectively. The injections were performed seven times, starting 1 or 24 hours after the tumor inoculation in the case of copper, and one hour after the inoculation in the cases of other metals. Both of bis-(dimethylglyoxime)-cobalt[6] and tris-(dimethylglyoxime)-cobalt[7] were also found to be non-effective on the tumor growth in the dose of 10 mg/kg/day.

3) ***The formation of the copper chelate and the antitumor activity.*** The aqueous solution containing dimethylglyoxime and cupric sulphate was neutralized with sodium hydroxide to pH 7.

As Figs. 2 and 3 indicate, the antitumor activity of dimethylglyoxime, in the presence of copper (3 mg/kg as cupric sulphate), increases as the dimethylglyoxime /copper ratio increases from 1 : 1 to 8 : 1 (M). In the ratio over 3 : 1, the treated mice survived over three times as the average survival days of control mice. The tumor growth was not observed, during the periods, in the treated mice. In the ratio of 1 : 1, dimethylglyoxime did not exhibit the antitumor activity even at the concentration of copper of 5 mg/kg, calculated as cupric sulphate.

Dimethylglyoxime itself shows the end absorption spectrum at 240–320 mμ. But it shows absorption spectrum at 400–750 mμ in the co-existence of the copper ion, forming a red brown solution. The absorption spectrum moves to a shorter wave length as the oxime/copper ratio increases from 0.5 : 1 to 4 : 1 (M). The spectrum

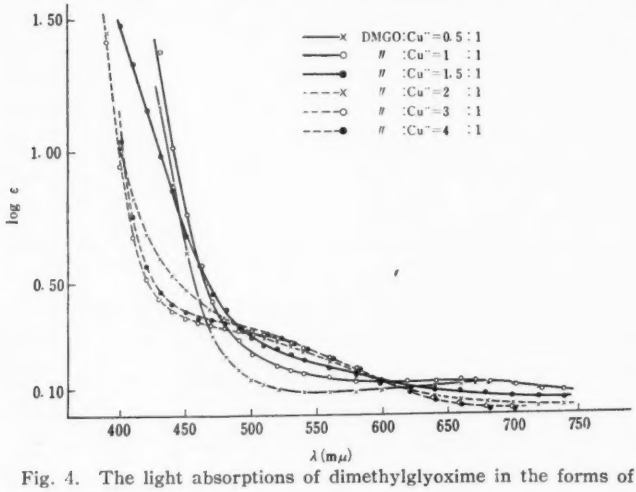


Fig. 4. The light absorptions of dimethylglyoxime in the forms of the copper chelates.

shows the fact that the chelate becomes stable in the ratio over 3 : 1 or 4 : 1 (M). These are shown in Fig. 4. It corresponds to the increase of the antitumor activity of the copper-dimethylglyoxime due to the increase of the ratio (Figs. 2 and 3). The formation of the chelate was ascertained by the observation of light absorption on the aqueous solution (pH 7). Beckman model DU spectrophotometer was used in this experiment.

The methanolic solution of dimethylglyoxime (3–4 M) was added to the aqueous solution containing cupric sulphate (1 M). After standing a day, it was neutralized with sodium hydroxide. The formed precipitate was filtered, and washed with ethanol or acetone. The analytical data of the deposited chelate showed the chelate to be a bis–(dimethylglyoxime)–copper.

Anal. found. : C, 32.52; H, 5.39; N, 19.21, %

Calcd. for $C_8H_{14}N_4O_4Cu$: C, 32.66; H, 4.79; N, 19.19

4) ***The preparatin of the solution containing the 'active copper-dimethylglyoxime'.*** The solution containing dimethylglyoxime (4 M) and cupric sulphate (1 M) was neutralized with sodium hydroxide to pH 7 after 24 hours standing.

The dose of the active chelate was expressed with that of cupric sulphate ($CuSO_4$ $5H_2O$) which had been used as a material. The molecular weights of bisi–(dmethylglyoxime)–copper and cupric sulphate (5 aq.) were 293.72 and 249.69, respectively. So, the expression of (2.5 mg/kg/day as cupric sulphate) may correspond to the dose of 3 mg/kg of the formed active chelate.

5) ***Antitumor activities of the 'active copper-dimethylglyoxime'.***

A) ***In vivo*** tests. a) Stained cell count method[4)8)9)]; the injurious effects of the chelates on the cell membranes of Ehrlich carcinoma. Usually, the normal cells may not be stained with eosin, as their cell membranes have the so-called selective permeability. Once the cell membrane be injured by the administration of some substances to break the selective permeability, the cell may be stained with eosin as observed in the dead cell.

The copper-dimethylglyoxime (25 γ/ml as cupric sulphate, ca. 30 γ/ml of the chelate) caused the increase of the number of stained cells with eosin in the ***in vitro*** contact test. Other well-known antitumor substances, such as sarkomycin, nitromin, and AZAN, required the concentrations of 15, 1–5 and 1 mg/ml, respectively, to show the same injurious action on the cells of the carcinoma. These are shown in Fig. 5 and Table 1.

b) T.T.C. Standing method, the inhibitory action of the chelates on the activity of dehydrogenase of Ehrlich carcinoma cells[4)9)]. The activity of dehydrogenase of Ehrlich carcinoma cells was inhibited by the copper-dimethylglyoxime (5–25 γ/ml as cupric sulphate), as shown in Table 2–a. The mixed solution of T.T.C. (2, 3, 5-triphenyl tetrazolium chloride) and methylene blue was put to use in this ex-

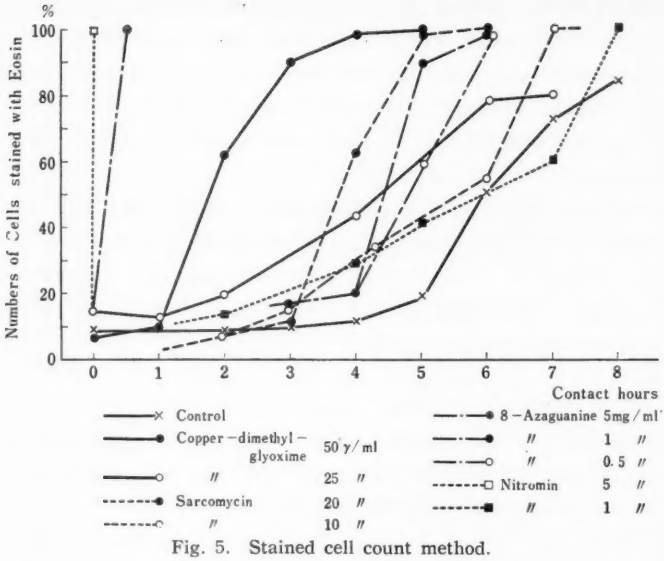


Fig. 5. Stained cell count method.

Table 1. Stained cell count method.

Compounds	Contact hours / (γ/ml)	Number of cells stained with eosin (%)				
		0	1	2	4	6
The active copper-dimethylglyoxime, prepared from dimethylglyoxime and cupric sulphate. (γ/ml as cupric sulphate)	100	15	75	100		
	50	10	69	85	96	96
	25	7	17	50	63	100
	10	8	11	19	64	82
	5	8	10	12	27	45
Bis-(dimethylglyoxime)-copper, prepared from dimethylglyoxime and cupric acetate.	50	10	43	82	100	
	10	12	32	42	55	82
	5	12	13	34	46	
	1	9	10	14	22	32
Control		10	10	11	20	23

amination. The formed formazan was extracted with ethyl-acetate, and was measured colorimetrically at 482 mμ. The amount of the formed formazan was decreased when the dehydrogenase activity of tumor cells was inhibited by the chelates.

B) ***In vivo*** test. a) Antitumor activity of the chelates on the ascites form of tumors. As shown in Figs. 1-a, 2, and 3, the increase of ascites fluides caused by the tumor growth was clearly inhibited by the administration of the chelates (2-5 mg/kg/day). The chelates administration prolonged the survival period over 3-4

Table 2-a. T. T. C. Standing method; The inhibitory action of the active copper-dimethylglyoxime on the activity of dehydrogenase (Ehrlich carcinoma)

Compound	γ/ml	T% (TTC-MB)
The active copper-dimethylglyoxime, prepared from dimethylglyoxime and cupric sulphate. (γ/ml calcd. as cupric sulphate)	50	100
	25	97
	10	81
	5	70
	2	59
Control		50

Table 2-b. T. T. C. Standing method; The inhibitory action of bis-(dimethylglyoxime)-copper on the activity of dehydrogenase (Ehrlich carcinoma)

Compound	(γ/ml)	T% (TTC)	T% (TTC-MB)
Bis-(dimethylglyoxime)-copper, having the planar and symmetrical structure. prepared from dimethylglyoxime and cupric acetate.	100	77	93
	60	35	78
	20	36	10
	10	36	10
	4	28	5
Control		25	5

times as that of the control mice. With even a single intraperitoneal injection at 3 days after the tumor inoculation, the chelate compound (5 mg/kg) exhibited the antitumor activity.

b) Antitumor activity of the chelates on the subcutaneous tumor of Ehrlich carcinoma. A conspicuous effect was observed with the hypodermic injection of

Table 3. *In vivo* test (5); The antitumor activity of the copper-dimethylglyoxime on subcutaneous Ehrlich carcinoma (1), expressed by measurement of long and short axises of tumors.

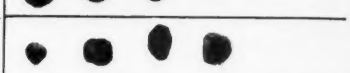
After transplantation	6 days	7 days	13 days	16 days	21 days
Control	+ (10 mm)	+	+	+	+
Copper-dimethylglyoxime	-	?	+	+	-
Cu··	—	+	+	+	+

Injections are initiated 2 days after the tumor transplant, once every day, 7 times. Control mice are treated with phsiological saline containing CMC (0.5%).

The chelates solution was prepared from dimethylglyoxime (2 M) and cupric sulphate (9.6 mg/kg/day, 1 M) with neutralization to pH 7.

Cupric sulphate (9.6 mg/kg/day) solution was prepared neutralizing it with sodium hydroxide to pH 7.

Table 4. *In vivo* test (6) The antitumor activity of the copper-dimethylglyoxime on subcutaneous Ehrlich carcinoma (2).

Compound	Initial date of injection after transplant	Dose (mg/kg /day)	Average weights of tumors (g)	Average weight ratio: $\frac{Treated}{Controls} \times 100$ (%)	Relative tumor size 20 days after transplantation
Control			1.82	(100)	
Copper-dimethylglyoxime injected 6 or 7 times, once a day, around tumors.	4	1.5	1.21	66	
	4	2.5	0.71	39	
	4	5	0.14	8	
	7	2.5	0.70	38	
	7	5	0.44	24	

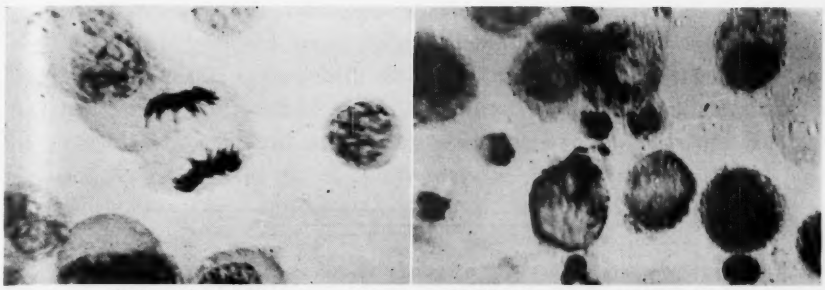
the active copper-dimethylglyoxime, as shown in Tables 3 and 4. The tumor growth was retarded showing the diminution in size of the tumors. 0.1 ml of the cell suspension of the physiological saline containing one million cells of Ehrlich carcinoma was transplanted subcutaneously into mice. The chelates was injected around tumors for 7 consecutive days, once a day, starting 4 or 7 days after the tumor transplantation. The progress of the tumors in the animals was recorded graphically by measuring them in two diameters with calipers, or measuring the tumor weight.

c) Effects on the cells of Yoshida sarcoma and Ehrlich ascites carcinoma. The destruction of the Yoshida sarcoma cells was observed with a single intraperitoneal injection of the chelate (50–5 mg/kg) into the tumor bearing rats, 4 days after the tumor transplantation. The number of tumor cells decreased during 1–24 hours after the injection, and the destruction was noticed in the nucleus, such as karyorrexis and karyolysis. After 48 or 72 hours, the proportion of tumor cells regained their normal states.

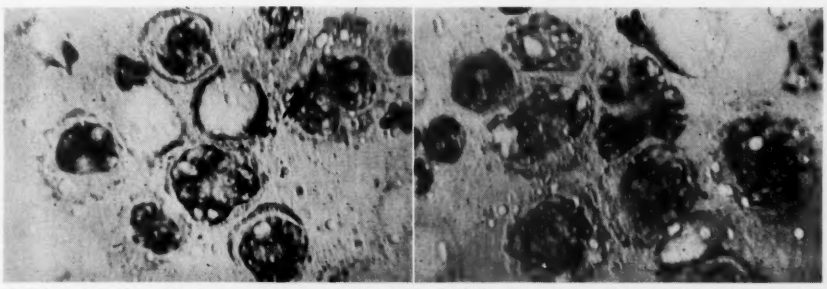
Almost the same destructive action was observed on Ehrlich carcinoma cells, as shown in Fig. 6.

6) *Acute toxicity*. Copper dimethylglyoxime was administered intraperitoneally into mice weighing 18 to 20 g. As shown in Table 5, the intraperitoneal acute toxicity test in mice indicates LD_{50} of 41 mg/kg.

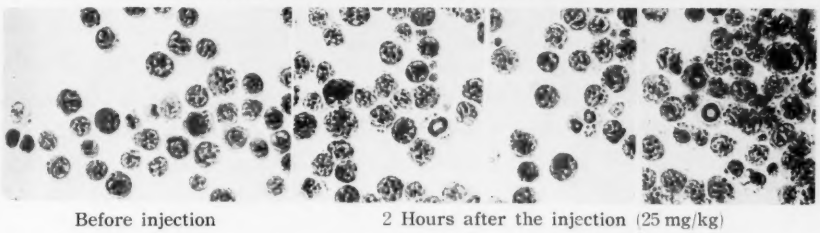
The LD_{50} on rats was determined by Dr. Hiroshi Sato.



Before the injection　　60 Minutes after the injection of the chelate Compd.



30 Minutes after the intraperitoneal injection　　60 Minutes after the injection (25 mg/kg)



Before injection　　2 Hours after the injection (25 mg/kg)

Fig. 6. *In vivo* test (7), Morphological findings of Ehrlich carcinoma cells in ascitic fluid after the injection of copper-dimethylglyoxime.

Table 5. Toxicity of a copper-dimethyl glyoxime. LD_{50}=41 mg/kg (mice, intraperitoneally)

Dose (mg/kg)	Number of mice dying			
	1st day	2nd day	3rd day	7th day
70	5/5			
50	0/5	2/5	4/5	5/5
30	0/5	1/5	2/5	2/5
10	0/5	0/5	0/5	0/5

They are; LD_{50}=75 mg/kg (rat, intraperitoneally)
MTD=50 mg/kg (rat, intraperitoneally)

7) ***Antitumor activities of copper-dimethylglyoxime; Bis-(dimethylglyoxime)-copper prepared from cupric acetate.*** Bis-(dimethylglyoxime)-copper was already synthesized by Tschugaeff[10] from dimethylglyoxime (2 M) and cupric acetate (1 M). The structure was determined by Ettore Bua[11] and Silvio Bezzi[12] on the basis of X-ray diffractian. It was found to be a symmetrical planar chelates. As shown in Fig. 7, the antitumor activity ***in vivo*** of this copper chelate was not remarkable.

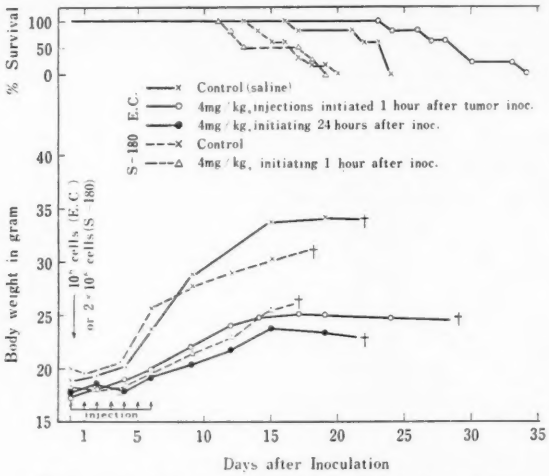


Fig. 7. ***In vivo*** test (8), Antitumor activites of Bis-(dimethylglyoxime)-copper on ascites forms of tumors.

It inhibited slightly the tumor growth of Ehrlich ascites carcinoma and sarcoma 180 in the dose of 4 mg/kg/day, when it was injected at one hour after the tumor inoculation. It showed the injurious action on the cell membranes of Ehrlich carcinoma, in the same dose with that of the 'active dimethylglyoxime' (Table 1), and required larger doses to inhibit the dehydrogenase activity (Table 2-b).

Anal. found : C, 32.86; H, 4.78; N, 18.76; Cu, 20.12%

Calcd. for $C_8H_{14}N_4O_4Cu$: C, 32.66; H, 4.79; N, 19.19; Cu, 21.59

Discussion

Leiter ***et al.***[3] observed the antitumor activity of o-phenanthroline and bipyridyl on sarcoma 37. Both of them have been known to be chelating agents for iron. And many o-phenanthrene and biphenyls did not show the activity. Even the two chelating agents, o-phenanthroline and bipyridyl, exhibited the antitumor activities near their maximum tolerable doses. If the non-ionic permeable molecules against cell membranes of tumors be prepared from the chelating agents, they may exhibit the better antitumor activities. As described already, the injurious action of α-ketoglutaric acid oxime, a chelating agent, on the cell membranes of tumors was

increased by forming its metal chelate compounds. The metal ions were assumed to mask the hydrophilic groups of the oxime to be easily permeated in tumor cells.

Dimethylglyoxime has also been known to be a chelating agent. It has been applied to the determination of nickel, because it forms quantitatively a stable water-insoluble chelates of bis-(dimethylglyoxime)-nickel from the solution containing the metal ion. The oxime has been indicated to form the chelate compounds with paladium[10)13)], copper[11)12)], cobalt[6)], and iron[14)]. Copper dimethylglyoxime was reported to be more soluble in water than the nickel chelates, as the length of Cu-Cu bond is larger than that of Ni-Ni bond. These chelates may easily permeate through cell membranes of tumors, consisting of the lipoid layer[5)], to exhibit the antitumor activity, as their hydrophilic groups are masked by forming the non-ionic, symmetrical, and planar molecules.

Of interest, only the copper-dimethylglyoxime was found to exhibit the antitumor activities, other chelates of dimethylglyoxime, such as bis-(dimethylglyoxime) -cobalt, or nickel, not showing the activity.

The chelate may permeate more easily into tumor cells than its original ligand, dimethylglyoxime and its copper moiety, as the hydrophilic hydroxy-groups and basic nitrogens of the oxime are masked by the copper. In tumor cells, the chelate may be dissociated to ligand and metal moieties to show the activity.

These points will be discussed in the following paper.

To know the active conformation of the 'active copper-dimethylglyoxime' solution, the bis-(dimethylglyoxime)-copper already synthesized by Tschugaeff[10)] was examined for its antitumor activities. It did not show a clear antitumor activity *in vivo*, but it caused the injurious action on tumor cells at the same concentration of the 'active chelate' in *in vitro* tests (10-25 γ/ml) (Table 1). The dehydrogenase activity of Ehrlich carcinoma cells was inhibited with the bis-(dimethylglyoxime)-copper in the dose of 60 γ/ml, and with the 'active copper-dimethylglyoxime' at the concentration of 15 γ/ml. The symmetrical planar bis-(dimethylglyoxime)-copper must be the main component of the active chelates, but the active conformation of the copper chelates, *in vivo*, may not be so simple. This point requires future study.

The other oximes, having structural relationships with dimethlglyoxime, were also examined for antitumor activities in the form of their copper chelates. They were biacetylmonoxime, acetylacetone dioxime, and others. That these oximes did not show the antitumor activities even in the presence of copper ion, may suggest the necessity of the ligand moiety of the copper-dimethylglyoxime. The possibilities to synthesize the more effective antitumor substances among chelates will be discussed in the following report.

Conclusion

Dimethylglyoxime (50 mg/kg/day) did not inhibit the growth of Ehrlich ascites carcinoma and Crocker sarcoma 180, but it (3–6 mg/kg/day) exhibited the antitumor activity in the presence of copper ion, though not in the presence of other metal ions, such as nickel, cobalt, zinc, iron, manganese, magnesium, and mercury.

In the presence of the copper ion, the dioxime was found to form a stable chelate at the physiological pH. As the dimethylglyoxime/copper ratio increases, the formation of the chelate becomes stable, and at the same time the antitumor activity of the chelate becomes more remarkable. Over the ratio of (3 or 4 : 1 M), it exhibited the clear inhibitory actions on the tumor growth.

The active chelate solution was prepared dissolving dioxime (4 M) and cupric sulphate (1 M) into physiological saline, standing 24 hours, and neutralizing with sodium hydroxide to pH 7. (1) The chelates (3–5 mg/kg/day) inhibited the increase of ascites fluides in the tumor bearing animals, and prolonged their survival periods over 3–4 times as that of the control mice. (2) Only 25 γ/ml of the chelate caused a destructive action on tumor cells, whereas the other antitumor substances, such as sarkomycin, nitromin, and AZAN required the concentrations of 15, 1–5, and 1 mg/ml, respectively, to show the same injurious action on tumor cells with the stained cell count method. (3) The chelates inhibited the dehydrogenase activity of Ehrlich carcinoma cells in the concentrations of 5–25 γ/ml. (4) The growth of the subcutaneous Ehrlich carcinoma was clearly retarded by the peritumoral injection of the chelates. (5) The destructive action on tumor cells of this chelate was also observed morphologically with Yoshida sarcoma and Ehrlich carcinoma. (6) The toxicity of the chelate was as follows: LD_{50}=41 mg/kg (mice, intraperitoneally), LD_{50}=75 mg/kg (rat, intraperitoneally), and MTD=50 mg/kg (rat, intraperitoneally).

To search for the active conformation of this chelate compound, the bis-(dimethylglyoxime)-copper having the symmetrical planar structure was examined for antitumor activities. It did not show the clear antitumor activities on tumors in ***in vivo*** test, though it injured tumor cells ***in vitro***.

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STUDIES ON ANTITUMOR ACTIVITIES OF OXIMES AND METAL CHELATE COMPOUNDS

III. COPPER COMPOUNDS[1]

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INTRODUCTION

Dimethylglyoxime, was found to exhibit the antitumor activity only in the form of its copper chelates, and not in those of other metals.[2] Forming the chelate compounds, the hydrophilic group of the dimethylglyoxime was assumed to be masked by the metal ion, and to become more permeable through cell membranes consisting of the lipoid layer.[3] The oxime may improve the metal penetration into tumors, or the metal may help the penetration of the oxime by forming the non-ionic permeable molecules.[2] The chelate compound may be disssociated into ligand and copper moicties, to show the antitumor activity.

It is interesting that the antitumor activity of the dimethylglyoxime was exhibitde only by its copper chelates. The copper may take a significant part in metabolic systems in tumor bearing animals.[4] Is the anitumor activity of the copper chelate due to the ligand moiety or the metal moiety? These points are further examined, as described in this paper.

Antitumor activities of other copper chelate compounds are also presented in this paper.

EXPERIMENTAL

1) ***In vivo*** tests: The antitumor activities were tested in the same way as in the previous papers[2][10] The injections of test solutions were initiated two hours after the tumor inoculation. The tumor strains used were Ehrlich ascites carcinoma and Crocker sarcoma 180.

2) The ***in vitro*** contact tests: The stained cell count method[5] and the T. T. C. standing method, were used as in the previous papers.[2][10]

3) The test solution: Each solution, containing a metal and chelating agents, was neutralized with sodium hydroxide to adjust to pH 7 after 24 hours standing.

4) The formation of chelate compounds and the destruction of a chelate, copper-dimethylglyoxime, by co-existing amino acids were observed with the use of a Beckman model DU spectrophotcmeter.

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5) The antimicrobial spectrum was examined with ordinary cylinder plate method. The minimum concentration of inhibition was measured on *Mycobacterium phlei* and *Mycob.* 607 in Kirchner's medium.[6]

6) The anti-nematode activity was examined on a *Diplogastor sp.* by a paper disk method. The concentration of inhibition (ppm) was measured microscopically with counting the survival ratio.

Results and Discussions

Chelating agents and the antitumor activities. Though it is still somewhat obscure how metals take parts in metabolic systems, in vivo, iron has been known to be contained in cytochrome, catalase, and peroxidase. Copper occurs in tyrosinase, laccase, ascorbic oxidase, butyryl co-enzyme A dehydrogenase, anb δ-aminolevulinic acid dehydrogenase. Copper is also presumed to take part in the iron-uptake into porphyllins.[7] The biologically active metals are responsible for the oxidazing-reducing functions of the enzymes, and they form chelates with amino acid residues of the protein in enzyme systems. Thus some compounds having the chelating ability are supposed to interfere with the utilization of metals by microorganisms, robbing them of the metals that are necessary for them to survive.[8] The chelating agents may also exhibit the antitumor activities for the same reason. In fact, α, α-dipyridyl and o-phenanthroline were indicated to show the antitumor activities against sarcoma 37.[9] Each of them has been known to have a selective chelating ability for iron. Many diphenyls and o-phenanthrenes, those that are not the chelating agents, were reported not to show the antitumor activity.[9] Even the two chelating agents, dipyridyl and o-phenanthroline, required the doses near the maximum tolerated to show their activities. Surely, tumors ***in vivo*** cannot be easily destroyed by the mere administration of these chelating agents.

In my previous paper, α-ketoglutaric acid oxime was indicated to be a chelating agent to form stable water-soluble chelates at the physiological pH.[10] This oxime was also reported not to exhibit a clear antitumor activities ***in vivo***. The destructive action of the oxime on tumor cells ***in vitro*** was indicated to be increased by forming its chelate compounds.[10] But, the chelate molecule was not fully non-ionic, as it carried the other non-reacted carboxyl group of the α-ketoglutaric acid oxime. If the more non-ionic molecules be prepared from oximes masking their hydrophilic groups by forming chelate compounds, the better antitumor activity may be observed, as the resulting non-ionic molecules will be easily permeated through cell membranes.[3]

As shown in Table 1, oximes having chelating abilities, such as biacetylmonoxime, acetylacetone dioxime, and others, did not show the antitumor activities on Ehrlich ascites carcinoma and Crocker sarcoma 180. Dimethylglyoxime, a chelat-

Table 1. Antitumor Activities of Oximes on Ehrlich Ascites Carcinoma (E.C.) and Crocker Sarcoma 180 (S-180). Intraperitoneal injection of test solutions initiated two hours after the tumor inoculation (one million cells /mouse). Seven times, once a day.

Substances	Daily dose (mg/kg/day)	Tumors	Average survival days, treated / controls	Suppression of the increase of ascites fluides
Glyoxime $HC(=NOH)-CH(=NOH)$	20	E.C.	20/17	—
	10	E.C.	21/17	—
	15	S-180	21/21	—
Acetylacetone $CH_3COCH_2COCH_3$	300	E.C.	25/22	—
	300	S-180	19/20	—
Acetylacetonedioxime $CH_3C(=NOH)CH_2C(=NOH)CH_3$	170	E.C.	16/17	—
	100	E.C.	19/17	—
Acetonylacetonedioxime $CH_3C(=NOH)CH_2CH_2C(=NOH)CH_3$	590	E.C.	18/14	—
	300	E.C.	16/14	—
Isonitrosoacetone $CH_3COCH=NOH$	20	E.C.	16/16	—
Methylglyoxime $CH_3C(=NOH)CH=NOH$	40	E.C.	16/16	—
Biacetylmonoxime $CH_3COC(=NOH)CH_3$	50	E.C.	16/16	—
	50	S-180	21/20	—

ing agent, was also reported not to exhibit the activity.[2]

The dimethylglyoxime was found to exhibit the antitumor activity only in the presence of a copper ion, and not in the co-existence of other metal ions.[2] The formation of the antitumor copper chelates of the dioxime was ascertained in the previous paper.[2] In this case, the antitumor activity of the chelate compound would not depend upon the chelating ability of the dimethylglyoxime to bind the extracellular metals that tumor cells required, as the dioxime was administered into tumor carrying animals in the form of its stable copper chelate. Rather, the chelating moiety of the chelate compound may react with intracellular metals during permeation in tumor cells to cause interference of naturally occurring metals which bind with their normal reactants.

The metal moiety and the ligand moiety of the copper-dimethylglyoxime for the antitumor activities. The hydrophilic groups of the dimethylglyoxime might be masked by forming the chelates, to be helpful to easily permeate in tumor cells through the lipoid layer of the cell membranes. The copper is grasped between four nitrogen atoms of the two molecules of dimethylglyoxime, and the two short hydrogen bonds are formed between four hydroxy groups of the two molecules.

If the antitumor activity of the chelate be due only to the formation of the permeable molecule, the dimethylglyoxime should also exhibit the activity in the existence of other metal ions, as the oxime has been reported to form stable chelates with copper, cobalt, nickel, iron and paladium. The bis-(dimethylglyoxime)-nickel [11-a)11-b)], copper[12)], cobalt[13-c)], iron[14)], and paladium[11-a)] were already indicated to be the planar and symmetrical chelates having no free hydroxy groups. Tris-(dimethylglyoxime)-cobalt[15)16)] was also synthesized showing the structure having no free hydroxy groups. In every chelate, the basic nitrogen atoms of the dioxime are masked with the metal ion, and the hydrophilic hydroxy groups are masked by forming the short hydrogen-bonds[11)16)] between them.

It is interesting that only the copper chelate of dimethylglyoxime exhibits the antiumor activity. Bis-(dimethylglyoxime)-nickel, bis-(dimethylglyoxime)-cobalt (10 mg/kg/day)[2)], and tris-(dimethylglyoxime)-cobalt (10 mg/kg/days)[2)] did not show the activity. Mercury has been known to be a very toxic metal, a strong ihhibitor for enzymes, as silver and copper. The mercury chelates also did not exhibit

Table 2. Effects on Ehrlich ascites carcinoma.

Substances	Daily dose. (mg/kg/day)	Average survival days. treated / controls	Suppression of body weight increase due to ascites fluide
Phthalocyanine[1)]	100	13/16	—
	50	14/16	—
	10	13/16	—
Cupric sulphate	12	16/20	
	9	17/20	
	6	20/20	—
Mercury-dimethylglyoxime	2)	17/16	—
	× 2	16/16	—
	× 4	16/16	—
Copper-biacetylmonoxime	3)	19/16	—
	× 2	19/16	—
	× 4	13/16	—
Copper-α-ketoglutaric acid oxime	4)	16/16	—
	× 2	17/16	—

1) Cyanine blue. Tokushu Chemical Co., LTD.
2) Dimethylglyoxime (9.2 mg/kg/day) and mercury nitrate (4.7 mg/kg) dissolved in physiological saline, and neutralized with NaOH to pH 7.
3) Biacetyl monoxime (16 mg/kg/day) and cupric sulphate (10 mg/kg) treated in the same way.
4) Prepared from α-ketoglutaric acid oxime (6.4 mg/kg/day) and cupric sulphate (5 mg/kg/day).

the activity, as shown in Table 2. These findings may show that antitumor activity of the copper dimethylglyoxime may be due to the copper moiety.

But, the copper chelate did not always exhibit the antitumor activity. As described later, copper phthalocyanine, copper biacetylmonoxime, and others did not exhibit the activity (Table 2). Copper was suggested to block the site of interaction between nucleic acids and proteins by Gale[17]. If the copper-dimethylglyoxime be introduced in tumor cells, the metal moiety of the copper-dimethylglyoxime can exhibit the inhibitory action.

The copper-dimethylglyoxime exhibited much more destructive actions on tumors than the original components, dimethylglyoxime and the copper. Cupric sulphate solution did not show the detectable antitumor activity ***in vivo*** in the dose of 12 mg/kg/day (Table 2). The necessary dose of the copper in the copper dimethylglyoxime, to exhibit the antitumor activity, corresponds to the dose of copper in

Table 3-a. Effects of copper chelates on the activity of dehydrogenase. (Ehrlich carcinoma cells)

Compounds	γ/ml	T% (TTC-MB)
Copper-dimethylglyoxime	50	100
	25	97
	10	81
	5	70
	2	59
Copper-biacetylmonoxime	100	100
	75	100
	50	98
	25	92
	10	67
Control	0	50

Table 3-b. Effects of copper α-ketoglutaric acid oxime on the dehydrogenase activity of Ehrlich carcinoma cells.

Compound	γ/ml	T%	
		(TTC)	(TTC-MB)
Copper-α-ketoglutaric acid oxime	50	87	76
	25	82	68
	10	76	42
	5	71	40
	2		22
Control	0	21	22

TTC : 2,3,5-Triphenyl tetrazolium chloride
MB : Methylene blue

cupric sulphate (3–5 mg/kg/day). The copper chelate of dimethylglyoxime inhibited the dehydrogenase activity of Ehrlich carcinoma cell at 25 γ/ml (Table 3-a). The cupric sulphate did not inhibit the dehydrogenase activity at the concentration of over 500 γ/ml. The injurious action of the copper chelate on tumor cells, to break the selective permeability of the cell membranes, was observed at 25 γ/ml. The inorganic copper compound required 10 mg/ml to show the same injurious effect[10]. The oxime moiety may improve the permeation of the active metal moiety into tumor cells by forming the permeable molecule. Thus, the ligand moiety may take a significant parts of the antitumor activity of the chelates, masking their hydrophilic groups with the metal ion to be permeable through cell membranes.

The antimicrobial spectrum of copper dimethylglyoxime shows the selective inhibitory action of the chelates on *Mycobacteria* (Table 4). Other copper chelates

Table 4. Antimicrobial Spectra (CAP Method)

Compounds	Diameters of inhibitory zone													
	Bacillus subtilis PCL-219	Sarcina lutea	Staphylo. aureus 209 P	Proteus vulgaris HX-19	Corynebacterium xerosis	Escherichia coli	Mycobacterium phlei	Mycob. 607	Candida albicans	Penicillium chrysogenum Q 176	Aspergillus niger NRRL-330	Piricularia oryzae	Saccharomyces cere.	Tolura utilis
Bis-(dimethylglyoxime)-copper[1]	‡	0	0	+	+	+	‡‡‡	‡‡‡	+	0	0	0	+	0
Dimethylglyoxime*	+	0	0	+	+	+	0	(+)	0	0	0		+	0
Copper-acetylacetonedioxime	0		0	+	+	+	※‡	‡‡	0	0	+	0	+	+
Acetylacetone dioxime	0	0				0	0	0	0	0	0		0	0
Copper-acetonylacetonedioxime	+		0	+	+	+	‡	‡‡	0	0	0	0	+	+
Acetonylacetone dioxime	+	‡				‡	‡+	‡	0	0	(‡)		0	0
Copper-α-ketoglutaric acid oxime	+		+	+	+	0		‡‡	0	0	0	0	0	0
$Cu(OH)_2$ [2] *	‡	0	0	+	+	+	‡+	‡	0	0	0		+	0
CuO *	+	0	0	0	+	0	‡+	‡	0	0	0	0	0	0
Cu_2O *	+	0	0	0	+	0	‡+	‡ ※	0	0	0	0	0	0

* suspension (2 mg of each compound/ml

+ : <15 mm ‡ : 35–45 mm

‡ : 15–25 ‡‡ : >55 mm

‡+ : 25–35

※: corresponds to streptomycin (8 units)

1) The minimum concentration of inhibition is 1 : 25000 against M. phlei and M. 607.

2) The minimum concentration of inhibition is 1 : 6000 and 1 : 7000 against M. 607 and phlei., resp.

having no antitumor activities, and inorganic copper compounds themselves, also exhibited the same selective inhibitory spectra. Dimethylglyoxime, the ligand moiety of the copper chelate, did not show the inhibitory action against *Mycobacteria*. These are shown in Table 4. It has been reported that the ionic copper in copper sulphate may be absorbed onto the surface of cells, such as of wheat bunt (*Tilletia*)[18] and *Alternaria tenus*[19]. Bodnar[20] suggested that the copper was able to replace calcium, magnesium, and hydrogen from the surface of spores. The alikeness of the antimicrobial spectra of the copper chelates, and the inorganic copper compounds, may be due to the same absorption onto the cells. The difference of the antitumor activities observed between copper chelates may be concerned with the nature of cell membranes of tumors. The lipoid layer of semipermeable membranes is surrounded by a protein layer to be hydrophilic in nature[3]. The water soluble copper compounds may permeate through this layer, but the permeation through the next lipoid layer may be difficult. The cupric sulphate did not show the antitumor activity as mentioned above. The water soluble complex

Table 5. Stained cell count method

Compounds	Contact hours / (γ/ml)	Number of cells stained with eosin, (%). 0	1	2	4	6
Copper-dimethyl-glyoxime[1]	100	15	75	100		
	50	10	36	69	96	96
	25	7	17	90	63	100
	10	8	11	19	64	82
	5	8	15	20	27	45
Copper-biacetyl-monoxime[2]	180	18	30	50	50	65
	100	12	23	42	46	50
	50	9	9	20	28	35
	10	8	8	19	24	25
	5	8	8	18	18	25
Control	none	7	9	18	18	26
Cupric-sulphate	500	17	29	40	55	80
	100	16	24	25	46	78
	50	9	24	24	34	40
Control	none	10	13	17	22	

1) 2) These chelates were prepared by neutralization, with sodium hydroxide, of the solution containing the oxime (4 M) and cupric sulphate (1 M) to pH 7 after 24 hours standing.

salt, copper phthalocyanine, did not exhibit the activity even in the large dose of 100 mg/kg/day (Table 2), though it contained much copper in the molecule.

Every ligand did not always show the antitumor activities. Dimethylglyoxime in the form of the copper chelates exhibited the activity, but other oximes, having structural relationships with it, did not show the clear antitumor activities in the presence of metal ions. They are glyoxime, biacetylmonoxime, acetylacetone dioxime, acetonylacetone dioxime, etc (Table 2 and Fig. 2).

The copper-biacetylmonoxime did not show the antitumor activity ***in invo***, even in the dose of 12 mg/kg/day (Table 2). As shown in Table 5, the copper chelates required over 180 γ/ml to cause the destruction of tumor cells, whereas the active copper dimethylglyoxime showed the same injurious action on tumor cells in 25 γ/ml. As shown in Table 3-a, the dehydrogenase activity of Ehrlich ascites carcinoma was inhibited by the copper dimethylglyoxime and copper biacetylmonoxime, in the same dose of 25 γ/ml. The former inhibited the enzyme action, and destroyed

Table 6. Inhibitory actions of copper chelates on a Nematode, Diplogastor sp., (Paper Disk Method).

Compounds	Concentration (ppm)			
	4000	2000	1000	500
Copper-dimethylglyoxime (1 : 4)	■	■	■	■
Copper sulphate	□	▯	▯	▯
Copper-acetylacetone dioxime (1 : 4)	□	▯	■	■
Copper-acetonylacetone dioxime (1 : 4)	□	■	■	□
Copper-α-ketoglutaric acid dioxime (1 : 4)	■	■	■	■
Copper-biacetylmonoxime (1 : 4)	□	▯	▯	■

■ 20 % Survival

the selective permeability of semipermeable membranes of tumor cells at the same concentration.

Copper-α-ketoglutaric acid oxime was already reported to cause the immediate injurious action on tumor cells at 25 γ/ml[10]. It did not show a clear inhibition of the dehydrogenase activity at 50 γ/ml (Table 3-b).

Thus, the antitumor activity of the copper chelates differs with the change of ligand moiety. The injurious action of the chelates on a nematode, ***Diplogastor sp.***, was also examined by the paper disk method. As shown in Table 6, the nematode was killed with cupric sulphate (1000 ppm). But the activity of copper ion decreased with forming chelate compounds with oximes. The formation of chelate compounds might increase the antitumor activity of copper moiety, and decrease the anti-nematode activity. The ligand moiety should be selected to prepare the chelates having antitumor activities.

Effects of naturally occurring chelators on the copper-dimethylglyoxime. During the penetration in the tumor cells, copper-dimethylglyoxime may exhibit the toxicity as a molecule, but, other wise, it must be dissociated into the components, copper and ligand moieties, to exhibit the inhibitory actions.

The affinity of metals for chelators was indicated to be in the following order, irrespective of the nature of their ligands:

$$Hg^{23\text{-a})},\ Pd^{21)} > Cu > Ni > Pb > Co = Zn > Cd > Fe > Mn > Mg$$

Then, the copper-dimethylglyoxime may not be dissociated by the presence of other metals except mercury which is a minor element ***in vivo***. In other words, the copper chelate may be stable ***in vivo*** for other co-existing metals.

The dissociation of the chelate may be preferably induced by the other co-existing, naturally occurring chelators, such as[22)23-a)] amino acid residues of proteins, peptides, natural purines, pteridines, riboflavins, phosphate, nucleotides, nucleic

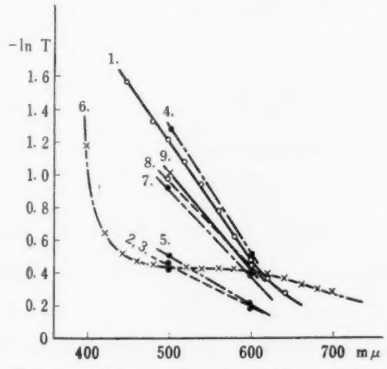

Fig. 1. Effects of Co-existing Amino Acids on a Copper-dimethylglyoxime.

No.
1 : Bis-(DMGO)-copper, prepared from the oxime and cupric acetate.
2 : 〃 + DL-Alanine
3 : 〃 + L-Aspartic acid
4 : 〃 + L-Glutamic acid
5 : 〃 + Glycine
6 : 〃 + L-Histidine
7 : 〃 + DL-Serine
8 : 〃 + DL-Threonine
9 : 〃 + Valine

acid, citric acid, and others. What the natural chelators grab the copper away from the copper-dimethylglyoxime, *in vivo*? A certain amount of each amino acid was added to the solution, containing copper-dimethylglyoxime, to observe the changes of the light absorption, due to the destruction of the chelate by the co-existing chelators. These are shown in Fig. 1. The destruction of the copper chelate with amino acids was found to be in the following order:

$$\text{Cystein (ppt)} > \text{Histidine} > \begin{matrix}\text{DL-Alnine}\\ \text{L-Aspartic acid}\\ \text{Glycine}\end{matrix} > \begin{matrix}\text{DL-Serine}\\ \text{DL-Threonine}\\ \text{DL-Valine}\\ \text{(DL-Glutamic acid)}\end{matrix}$$

The relative degree of the dissociation, and toxicity of copper complexes of amino acids, were indicated to be almost in the same order[8]:

Glut. acid>DL-Alanine>DL-Valine>Leucine>Asp. acid>Cystein

Table 7. Effects of co-existing amino acids on the bis-(dimethylglyoxime) copper.

Compounds	−1 n T		Diameters of inhibitory zone	
	500 mμ	600 mμ	M. phlei	Mycob. 607
bis-(dimethlglyoxime)-copper[1]	1.23	0.48	++	++
+DL-alanine (1.8 mg)[2]	0.51	0.21	++	+++
+L-aspartic acid (2.7 mg)[2]	0.41	0.20	0	++
+Cysteine (3.2 mg)[2]	ppt.	ppt.	+	++
+L-glutamic acid (4.3 mg)[2]	1.29	0.50	++	+++
+glycine (1.5 mg)[2]	0.45	0.19	+	+
+L-histidine (4.2 mg)[2]	0.44	0.40	0	+++
+DL-serine (2.4 mg)[2]	0.92	0.39	++	++
+DL-threonine (2.4 mg)[2]	0.98	0.46	++	+++
+valine (2.1 mg)[2]	1.05	0.41	++	+++
Copper-DL-alanine[3]			(++)	++++
Copper-L-aspart. acid[3]			(++)	++++
Copper-L-glutam. acid[3]			(++)	++++
Copper-L-histidine[3]			0	++++
Copper-DL-threonine[3]			(++)	++++
Copper-L-valine[3]			(++)	++++

1) 5 mg/ml

2) Each amino acid was added to the solution containing bis-(dimethylglyoxime)-copper (5 mg/ml), and neutralized with sodium hydroxide to pH 7.

3) The same mg of each amino acid with that of 2) was added to the copper sulphate solution (5 mg/ml), and neutralized to pH 7.

The majority of the amino acid was shown to have the similar constants, except cystein and histidine which form the most stable chelates with metals. The order of the copper–dimethylglyoxime, to be destroyed with amino acids, was almost the same as that of the stability constants of amino acids. The most destruction of the chelate was caused by co–existing cystein and histidine. These are also shown in Table 7. The antimycobacterial activity of the chelates against *Mycob. phlei* was decreased by co–existing amino acids, such as histidine, aspartic acid, and others. The increase of the activity against *Mycob.* 607 with the addition of histidine was observed, showing the formation of copper–histidine which had an inhibitory action on *Mycob.* 607, but not on *Mycob. phlei.*

The metal binding of the protein was assumed to occur mainly on the cystein and histidine residues[22]. The cysteine was assumed to be responsible for seizing the copper from copper oxinate[8)23)]. The metal moiety of the copper–dimethylgly-

Table 8. Effects on Ehrlich ascites carcinoma

Substances	Average survival days, $\frac{\text{treated}}{\text{controls}}$	Suppression of body weight increase due to ascites fluid
dimethylglyoxime–Cu–α alanine	13/19	−
〃 –Cu–β alanine	21/28	−
〃 –Cu–arginine	17/28	−
〃 –Cu–aspartic acid	20/28	−
〃 –Cu–cysteine	22/28	+
〃 –Cu–cystine	20/19	−
〃 –Cu–l–glutamic acid	28/28	−
〃 –Cu–d–glutamic acid	21/19	−
〃 –Cu–glutathione	19/19	−
〃 –Cu–glycine	24/28	−
〃 –Cu–histidine	23/28	−
〃 –Cu–leucine	21/28	−
〃 –Cu–lysine	25/28	−
〃 –Cu–methionine	22/28	+
〃 –Cu–norvaline	20/28	−
〃 –Cu–phenylalanine	26/28	−
〃 –Cu–proline	22/28	−
〃 –Cu–serine	21/28	−
〃 –Cu–tryptophane	29/28	−
〃 –Cu–threonine	28/28	+
〃 –Cu–tyrosine	14/19	−
〃 –Cu–valine	21/28	−

Equimolecular of cupric sulphate (2.5 mg/kg/day), dimethylglyoxime, and each amino acid were dissolved in physiological saline and neutralized with NaOH to pH 7. Intraperitoneal injections of the test solutions were initiated two hours after the tumor inoculation (one million cells/moue). Seven times. daily. Each group consisted of 5 mice, 18–20 g body weight.

oxime may also be grabbed by the same amino acids residues, ***in vivo.***

Copper complexes of amino acids on the performance of the antitumor activity. The solution containing dimethylglyoxime, copper ion, and amino acid in the ratio of 1 : 1 : 1 (M) was prepared at pH 7, and examined for their antitumor activities on Ehrlich ascites carcinoma. These are shown in Table 8. Among 22 amino

Table 9. Effects on Ehrlich ascites carcinoma

Substances	Daily dose	Average survival days, treated/cntrols	Suppression of body weight increase due to ascites fluid
Dimethylglyoxime–Cu–threonine	A	22/12	++
〃 –Fe–threonine	A	20/12	+
〃 –Co–threonine	A	13/12	−
〃 –Cu–cysteine	A	13/12	−
〃 –Fe–cysteine	A	19/12	−
〃 –Co–cysteine	A	13/12	−
〃 –Cu–d–glutamic acid	B	28/13	++
〃 –Cu–l–glutamid acid	B	27/13	++
〃 –Cu–tryptophane	B	25/20	−
〃 –Cu–histidine	B	20/20	−
〃 –Cu–aspartric acid	B	26/20	−
〃 –Cu–serine	B	25/20	±
Copper–dimethylglyoxime (1 : 1)	C	19/20	−
Copper–dimethylglyoxime (2 : 1)	C	23/13	++
Copper–dimethylglyoxime (4 : 1)	C	34/13	+++
Cu–threonine (1 : 1)	C	18/16	−
Cu–l–glutamic acid (1 : 1)	C	19/16	−

A: The physiological saline containing cupric sulphate (2.5 mg/kg/day, 1 M), dimethylglyoxime (2 M), and each amino acid (2 M) was neutralized with NaOH to pH 7.

B: The solution containing equimolecular cupric sulphate (5 mg/kg/day), dimethylglyoxime, and each amino acid was neutralized to pH 7.

C: The solutions were prepared from cupric sulphate (5 mg/kg/day).

acids, the copper dimethylglyoxime (1 : 1) showed the slight activity in the co-existence of methionine and threonine. Further examinations were performed as shown in Table 9. A clear antitumor activity was observed with the complexes consisting of dimethylglyoxime, copper, and threonine or glutamic acid (1 : 1 : 1). The solution, containing dimethylglyoxime, copper, and cystein, histidine or others, did not show a clear activity. The copper–dimethylglyoxime (1 : 1), copper–threonine (1 : 1) and copper–glutamic acid (1 : 1) did not show the detectable suppression of the increase of ascites fluids. Thus, the active chelate must be consisted of the oxime, copper, and some each amino acid or a further oxime.

As suggested by Albert,[24] the formation of somewhat unusual 1 : 1 chelate (Cu^{+} dimethylglyoxime) may cause the poor permeation, carrying the charge. The rapidly-penetrating 1 : 2 complex of copper-dimethylglyoxime, may be dissociated

intracellularly into the unusual 1:1 chelate, to exhibit the antitumor activity *in vivo* and the latter complex may react with the naturally occurring chelators to form the (1:1:1) copper complexes, including the amino acid residues, *in vivo*. The fact that the threonine-containing complexes exhibited the antitumor activity in its copper complex, and not in its ferrous and cobalt complexes seemed interesting (Table 9).

Antitumor examinations of copper chelates.

As described already, dimethylglyoxime exhibited a clear antitumor activity by forming the stable chelate compound with copper. The hydrophilic nitrogens of the dioxime are masked with the metal ion, forming a planar and symmetrical structure. The hydrophilic hydroxy groups are also masked by forming short hydrogen-bonds between the two ligand moieties.

1) The copper chelate of biacetylmonoxime did not show the antitumor activity even in the higher dose than that of copper dimethylglyoxime in *in vivo* (Table 2) and *in vitro* tests (Table 3 and 5). The symmetrical structure of the latter chelates may be necessary for the exhibition of the activity.

2) Copper glyoxime did not show the activity (Fig. 2). It may form the symmetrical structure. The terminal lipophilic methyl group adjacent to the isonitroso group of the dimethylglyoxime may be necessary for the activity.

3) Acetylacetone dioxime or acetonylacetone dioxime was also found to form the copper chelates. They did not show the activity (Figs. 2, and 3). The seven-membered ring of the latter chelate might be unstable, but the former chelate should have a stable six-membered ring. The hydroxy groups of the copper acetylacetone dioxime might hinder the exhibition of the activity.

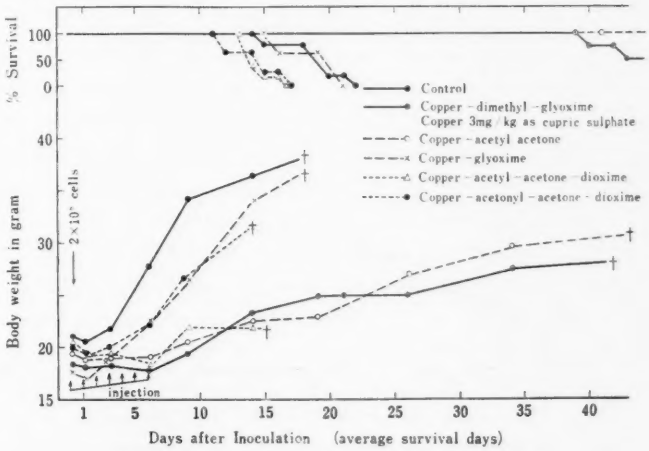

Fig. 2. *In vivo* test (1), (Crocker sarcoma 180)

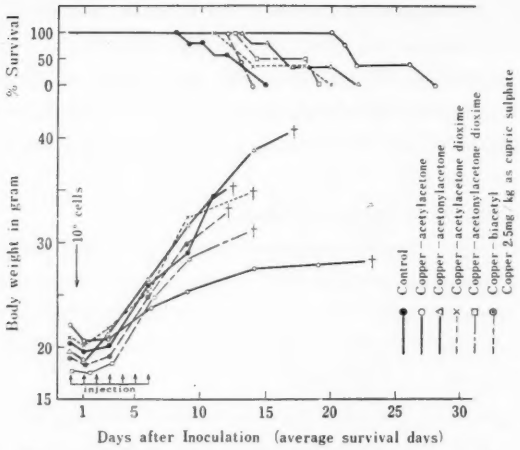

Fig. 3. *In vivo* test (2), (Ehrlich ascites carcinoma).

4) As the compounds having no hydroxy group, the corresponding carbonyl compounds were examined for their antitumor activities in the presence of copper ion (Figs. 2, and 3). Acetylacetone was found to show the activity in the presence of coppper. The crystal of copper-acetylacetone (4 mg/kg/day) also exhibited the antivity (Fig. 4). The average survival days was prolonged twice of that of the control mice. The original acetylacetone did not show the activity even in the dose of 300 mg/kg/day against Ehrlich carcinoma and Crocker sarcoma 180 (Table 1). Both of biacetyl and acetonylacetone did not exhibit the antitumor activities even

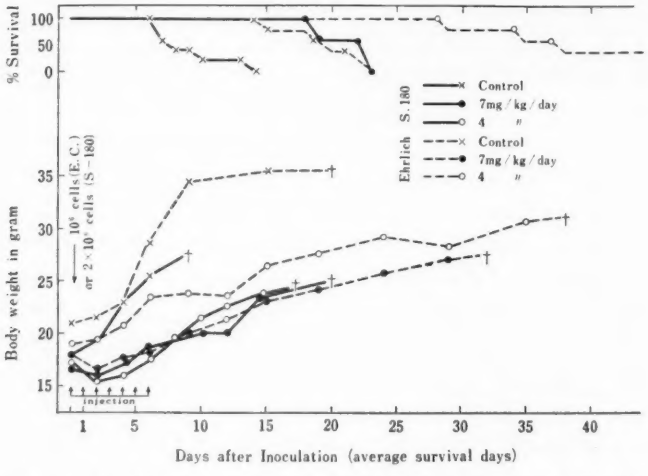

Fig. 4. *In vivo* test (3), Antitumor activities of bis-(acetylacetone)-copper on Ehrlich ascites carcinoma and Crocker sarcoma 180.

in the presence of the copper ion (Fig. 3).

Conclusions

Metals may form chelates with amino acid residues of the protein in enzyme spstems, and may be responsible for their oxidizing-reducing functions. Thus the chelating agent was supposed to exhibit the anitumor activity by robbing cells of the necessary metals.

To exhibit the clear activity, the hydrophilic groups of the chelating agents should be masked by forming their metal chelate compounds to be more permeable through cell membranes. The metal chelate compounds of α-ketoglutaric acid oxime or dimethylglyoxime exhibited the inhibitory activities on tumor cells, but the original chelating agent did not exhibit the activity. Further dimethylglyoxime exhibited the antitumor activity in the form of copper chelates. If the antitumor activity of the chelate compounds be due only to the formation of the permeable molecule, the dimethylglyoxime should exhibit the activity in the presence of other metal ions.

Copper was suggested by Gale to block the site of interaction between nucleic acid and proteins. If the copper chelate compounds be introduced into tumor cells, the metal moiety of the chelates may exhibit the inhibitory actions. But not all the copper chelates always exhibit the antitumor activity. The ligand moiety may take a significant parts in the antitumor activity of the chelates, by improving the permeation of the active metal moiety into tumor cells.

The injurious action of the chelates on cell membranes of tumors, and the ***in vivo*** antitumor activity, differed with the change of ligand moiety, but their inhibitory action on the dehydrogenase activity of cells was not changed. These were observed with copper-dimethylglyoxime and copper-bisacetylmonoxime. De Long ***et al.***[25] observed a decreased calcium content which is peculiar to tumor tissues, and is partially responsible for a loss of adhesiveness of the surface of tumor cells. Ambrose ***et al.*** (1956)[26] observed a marked difference in the electrophoretic motility between tumor and normal cells. As suggested by Brachet[27] (1957), if the increased motility of the cancer cells is to be attributed to different properties of the cell membrane, the antitumor substances, having specific inhibitory actions by breaking exclusively the selective permeability of cancer cells, may be formed. Some copper chelates, having the adequate ligand moiety, may exhibit the specific inhibitory action on tumor cells.

After the permeation into the tumor cells, the copper dimethylglyoxime may be dissociated into copper and ligand moieties to exhibit the inhibitory actions. The dissociation of the chelates may be induced by the naturally occurring chelators, having stronger avidities to copper than the oxime. These may be the cystein or

histidine residue of proteins in tumor cells. The rapidly penetrating 1:2 complex of copper-dimethylglyoxime may be dissociated into the unusual 1:1 chelate to exhibit the antitumor activity within tumor cells. What natural chelators would be responsible for the reaction seemed interesting.

Among other copper chelate examined, the copper acetyl acetone exhibited some antitumor activities.

Summary

The chelate compounds of oximes and carbonyl compounds were examined for their antitumor activities on Ehrlich carcinoma and Crocker sarcoma 180.

The activity of the copper dimethylglyoxime was discussed in the terms of ligand and metal moieties, or the extracellular and intracellular activities. The possibility of preparing other active copper chelates, having the selective inhibitory actions on tumor cells, by changing the ligand moiety, was suggested.

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LIVER CATALASE ACTIVITY IN GASTRIC CANCER PATIENTS, IN RELATION TO THE CLASSIFIED TYPES OF GASTRIC CARCINOMA AND PATHOLOGICAL CHANGES OF LIVER

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INTRODUCTION

Many reports have been made concerning malignant growths and their general effects on the host. From the reports of Rosenthal (1) and Brahn (2), it is also known that liver catalase activity decreases in animals bearing tumors. Recently Greenstein and his coworkers reported that decrease of liver catalase activity was specifically due to the presence of a neoplasm. Their reports show that hepatic catalase activity is similarly reduced in animals inoculated with any kind of malignant tumor, either cancer or sarcoma (3–8). Surgical removal of these tumors from the animals, restored the liver catalase activity to the normal level (3, 7). Pregnancy or inoculation of embryonic tissue failed to impair the activity (8). Catalase is present in various organs, such as the liver, spleen, kidneys and erythrocytes. Liver catalase was however reduced in tumor-bearing animals. From these facts, Greenstein and others maintained that decrease of hepatic catalase activity was specifically caused by the development of malignant tumors. Decrease in catalase activity was apparently not due to the presence of possible inhibitors, but to a decreased quantity of total catalase per se (9). It is of great interest to know why the level of catalase was specifically reduced only in the liver and not in other organs. Despite efforts made by many workers, this problem has not yet been solved. In the light of laboratory studies on animals there are several ways of looking at and explainining this phenomenon of the decrease of liver catalase activity in patients with gastric cancers. It has been noted that there are : [1] a nutritional deficiency in patients with gastric cancers (because of a low protein diet and hunger) and also secondary changes in the liver caused by certain toxic substances (3, 10–13) : [2] inadequate iron-absorption (utilization) due to a disorder in the gastric mucosa and its accompanying hypoacidity or anacidity (14–18) : [3] a secondary effect caused by a disorder of the endocrine glands (e.g. adrenal cortex) due to the development of the cancer (24) : [4] disturbed iron-utilization or dis-

turbed synthesis of the enzymic protein, which is attributable to the toxins secreted from the cancer tissue (designated "toxohormone" by Nakahara and his coworkers (20)) : and[5] translocation of iron in cancer (Greenstein (21)).

As we had many opportunities to perform laparotomies on patients with gastric cancers, we gave special consideration to this problem. A minute pathological investigation was carried out on samples of stomach. Also an assay was made of the catalase activity and a histological and histochemical study was made of specimens of liver excised from patienets with gastric cancer. Correlations among these three investigations were studied.

Materials

The specimens of stomach used were taken from patients with gastric cancer, gastric ulcers, chronic atrophic gastritis, and chronic pancreatitis, etc., in our clinic from August, 1954, to October, 1955. Liver specimens were excised from the front central part of the left lobe of the liver during operations.

Methods

Liver slices excised during the operation were divided in two. One half was used for the histological and histochemical studies, and the other for measurement of the catalase activity. In parallel with the above examinations, minute histological studies were made on the resected stomach.

1) Histological examination of the resected stomach specimens :—Fresh specimens were investigated macroscopically. Special care was taken over measurements. Careful observation was made on the attitude of the gastric mucosa, infiltration of the gastric walls and perigastric tissues, as well as metastasis in the lymphnodes. After fixation with formalin, two or three long slices along the major and minor curvatures were made inclusive of the pyloric and cardial ends, as well as the lesion. They were stained with hematoxilin-eosin, and by van Gieson and also impregnated with silver, and investigated in detail. Each lymphnode was studied histologically for the existence of metastasis.

2) Histological and histochemical studies on liver specimens :—The excised liver was not only stained with hematoxilin-eosin, azan and impregnated with silver for the study of its general structure, but was also examined histochemically with Sudan IV, PAS, pilonin-methyl-green and Heidenhain's stains.

3) Assay of liver catalase activity :—a) Outline of Assay method. In accordance with Euler-Josephson's method (22), an appropriate amount of phosphate buffer, enzymic solution and distilled water, were placed in a flask (capacity 50 ml) which was kept in an ice bath. When the contents of the flask reached 0°C, the substrate, hydrogen peroxide, at optimum concentration, (which had also been kept in the ice bath at 0°C) was added. The flask was shaken gently. Exactly

five minutes later 40% sulfuric acid (2.5 ml) was added to terminate the reaction. In the control experiment, the same amount of sulfuric acid was added initially, and the substrate was added later. Then, with potassium permanganate solution the hydrogen peroxide remaining was titrated and the amount of the substrate broken down was finally calculated.

b) Preparation of the enzymic solution and assay conditions. A fresh liver specimen was ground with the same amount of sea sand, and then extracted with 1/100 M. phosphate buffer at pH 6.8. (500 ml per gm of liver slice) for one hour. The solution was centrifuged at 3000 r.p.m. for 5 minutes. The supernatant was then diluted one in 2000, 3000 and 4000 and used for the assay.

From the results of the above preliminary examinations, the following conditions were fixed for the assay of catalase activity.

Substrate (H_2O_2 solution)	: 1 ml (4.5 mg)
1 in 3000 enzymic solution	: 2 ml
1 M. phosphate buffer solution (pH 6.8)	: 1.5 ml
Distilled water	: 10.5 ml
Total volume	: 15 ml

Reaction temperature: 0°C, Incubation time: 5 minutes, pH: 6.8

The velocity constant under these conditions was 0.03818.

Results

1) *Histological findings in the stomach and catalose activity:—*

Gastric specimens were classified histologically into a non-cancer group (gastric & duodenal ulcers, chronic atrophic gastritis) and a cancer group. In the latter group the following points were particularly considered; macroscopically: whether a tumor or ulcer was formed in the lesion; or whether a submucous lesion was dominant. Microscopically: the grade of anaplasia of the cancer cells; disorders of gastric mucosa distant from the lesions; changes of 'muscularis mucosa' at the border of the ulcers; and the existence of a gap in the muscle layer. From the precise analysis of these points, the cancer group was further classified according to Prof. Kuru's classification (26, 27, 28) into 4 types of gastric cancer. Type A is 'bulky', and supposed to be developed upon a gastric polyp (which is designated as polyp-cancer). Type B is 'ulcerative', and supposed to be developed upon a penetrating gastric ulcer (ulcer-cancer). Type C is 'infiltrative', and supposed to be developed on a chronic gastritis with atrophic changes of gastric mucosa (gastritis-cancer). Type D is 'submucous', and supposed to be developed upon a pre-existing aberrant tissue (especially the aberrant pancaeas) in the submucous tissue (aberrant-cancer).

Catalase activity of the liver was assayed in both groups as shown in Tables 1 and 2.

Table 1. Non-Cancer group

No.	Name	Age & Sex	Diagnosis	Findings of stomach specimen				Gast. acid (HCl)	Liver catalase activity
				Mucosal Changes	Extent of mucosal atrophy	Submucosal Changes	Miscellaneous		
1	D.N.	40 f	chr. atr. gastritis	folds few, metaplasia, Paneth cell.	###	conn. tiss. proliferation		12	40
2	M.J.	24 m	〃	thin	###	〃		15	39.5
3	Y.M.	36 f	〃	thin, metaplasia Paneth cell	###	〃 vessels & red cells many	partly hypertrophy, small necrosis	34	41.3
4	M.T.	56 f	〃	thin, metaplasia, superficial ulcer, partly cut	###	〃	muscular layer regular, lymph. tiss many		22
5	M.S.	32 f	〃	metaplasia, chromatin many	###	〃		6	36
6	U.S.	30 m	〃 granulous	thin	##	〃 granulous	healed ulcer, lack of muscularis	85	10.5
7	K.D.	51 m	〃	folds few, metaplasia	###	〃	superficial necrosis	7	70
8	S.K.	42 m	gastritis		##	〃		—	41
9	M.T.	42 m	antrum gastritis	thin, metaplasia	###	replaced by conn. tissue	small ulcer	11	30.9
10	F.K.	38 m	antrum atrophic gastritis	metaplasia	##	〃		—	59
11	H.G.	38 m	〃	thin, folds few, metaplasia	++	conn. tiss. proliferation	small ulcer healed	79	23
12	C.K.	49 m	〃		###			6	45
13	T.K.	33 m	gastric ulcer & antrum gastritis	granulous	##	〃	lack of muscularis at the site of ulcer (2.5×2.8) superficial necrosis	58	40.5
14	A.K.	45 m	〃	thin, metaplasia	###	replaced by conn. tissue	hypertrophy, lack of muscularis	9	39
15	Y.M.	63 f	〃	thin, metaplasia	##	conn. tiss. proliferation	ulcer formation	30	36.8
16	F.K.	57 m	〃	metaplasia	++	〃	〃	45	45.3
17	H.R.	49 m	〃	atrophy marked, metaplasia	###	〃	bleeding in ulcer, lack of muscularis	40	30.8
18	F.J.	31 m	gastric ulcer	thin	++	〃	ulcer formation (1.4×1), fresh necrosis	58	37.5
19	O.H.	51 m	〃	metaplasia, granulous	++	〃	lack of muscularis at the site of ulcer' regenerated epithelium	85	26
20	U.E.	47 m	〃	no metaplasia	+	〃 edema	ulcer formation (2×1), lack of muscularis	31	41.7
21	S.M.	51 m	〃		+		big ulcer (6×6) muscularis remained		15.5
22	I.W.	19 m	duodenal ulcer	fresh necrosis	##	conn. tiss. proliferation	thick muscularis	33	31
23	O.D.	28 m	〃		++	〃		—	33
24	T.K.	27 m	〃	atrophy	+			46	49
25	I.N.	30 m	〃		++	〃		15	43
26	M.T.	43 m	cholelithiasis						34.9
27	M.T.	50 m	〃						23.6
28	O.K.	59 f	〃					29	7.4
29	Y.M.	31 m	chronic pancreatitis					67	21.8
30	F.J.	28 m	intestinal polyposis				tall polyps arranged regularly, benigne		29

Table 2. Cancer group

No.	Name	Age & Sex	Diagnosis	Findings of stomach specimen				Lymphnodes metastasis	Gastric acidity (HCl)	Liver catalase activity
				Changes distant from lesion			Changes of lesion			
				Mucosa		Submucosa	size, infiltration, etc.			
				extent of atr.	other changes					
1	Y.M.	49 m	gastritis-cancer	###	diffuse atrophy	conn. tiss. proliferation	big (15×17×3), to serosa, no defect of muscularis	##	–	40.0
2	Y.M.	69 m	〃	###	〃	〃	big (11×8×2), to pancreas & duodenum	##	36	8.6
3	H.R.	39 m	〃	###	thin	〃	big (9×8×3), to muscularis, no defect of muscular	##	–	20.3
4	K.W.	50 m	〃	##	metaplasia	〃	small (4×2×1), to muscularis	##	17	20
5	S.N.	47 f	〃	###	fresh necrosis bleeding metaplasia	hypertrophy	big (9.5×12×2), to serosa	–	–	34
6	Y.S.	54 m	〃	##	metaplasia		big (7×3.8×3), to submucosa, muscularis remained	–	6	22 3
7	O.H.	64 m	〃	###	thin, diffuse atrophy	conn. tiss. slightly proliferated	big (6 5×6×4), to muscularis		18	25.4
8	H.I.	58 m		###			big (5×9×2), initial stage	–		28.8
9	S.K.	56 f	〃	##	metaplasia	conn. tiss. proliferated	big (6×4×3), to serosa	##	25	25
10	W.A.	65 m	〃	###	thin	〃	big (9×5×2), to serosa, no defect of muscularis	##	–	19.6
11	T.J.	72 m	〃	##	metaplasia marked	replaced by cancer cell	big (6.5×8×3), to serosa	##	16	30
12	Y.U.	47 f	〃	##	thin, metaplasia	conn. tiss. proliferated	small (3.5×1.5×2), to serosa, muscularis regular	##	19	26.8
13	T.k.	46 m	〃	###	thin, metaplasia, diffuse regressive changes	conn. tiss. proliferated in deep layer	big (10×6×3), to serosa & lymph. nodes, muscularis traceable	###	–	24.1
14	S.G.	56 m	polyp-cancer	##	hypertrophy, metaplasia	conn. tiss. proliferation	big (6×12×3), to serosa	##	28	21
15	N.K.	55 m	〃	###	metaplasia	〃	big (6.8×9 5×2), to muscularis, muscularis mucosa turned up (into the lumen at the border of ulcer)	+	–	34.4
16	J.N.	61 m	〃	###	thin metaplasia	〃	big (8×6×3), to muscularis, muscularis mucosa turned up	##	–	24.6
17	I.M.	67 m		###	metaplasia	〃	big (7×5×2), to muscularis & serosa, muscularis mucosa turned up	+	–	8
18	T.K.	51 m	〃	###	partly elevated due to metaplasia	conn. tiss proliferated in deep layer	big (9×5×3), to serosa, musculaaris mucosa turned up, one end of ulcer was cancerous	+	–	26
19	I.W.	62 f		###	metaplasia		big (8×5×3), to muscularis, muscularis mucosa turned up, muscularis regular		–	28
20	Y.M.	68 m	〃	###	thin, metaplasia	conn. tiss. proliferated	big (5×7×3), to muscularis, muscularis mucosa turned up, muscularis traceable	##	8	23
21	N.O.	64 m	〃	###	superficial necrosis, metaplasia	〃	big (6.5×8×2), to serosa, muscularis mucosa turned up,	##	–	26
22	M.T.	60 m	〃	###	thin	replaced by conn. tiss	two lesions, medium (5×3.5×2,5×3×2), to serosa, muscularis turned up, regular	–	26	39.6

23	T.R.	55 m	〃	##	glands disappeared, metaplasia	conn. tiss. proliferated	big (15×6.5×2) to perigastric tiss., muscularis turned up	##	–	26
24	N.O.	59 f	〃	#	metaplasia	〃	big (7×4×3), to muscularis, muscularis turned up	##	20	29
25	K.M.	56 m	〃	#	metaplasia	〃	big (6.5×4×2), to muscularis, muscularis turned up, small polyp	+	–	38.8
26	M.S.	60 f	〃	##			big (7×10×5), to outside of serosa, muscularis turned up	+	–	11.3
27	A.S.	50 f	〃	–~+			medium (4×5.5×2), to muscularis, muscularis turned up, muscularis regular	–	10	31
28	K.S.	52	ulcer-cancer	–	hypertrophic change	no defect of muscularis	big (6.5×7×3), to muscularis, muscularis mucosa turned down (to the base of ulcer at the border of ulcer)	–	93	34.9
29	S.B.	60 m	〃	#	partly hypertrophy, necrosis	conn. tiss. proliferated, hypertrophy	medium (4×4×2), to serosa, muscularis turned down, cancer cell filled lymph. lumen	##		2.95
30	S.B.	61 f	〃	##	thin metaplasia	replaced by conn. tiss. in deep layer	big (10×6×2), to serosa, muscularis once turned up and then down, cancer cell in muscularis	##	29	48
31	S.G.	54 m	〃	##	thin, glands disappeared, metaplasia	conn. tiss. proliferated	big (9×6×2), to serosa, superficial ulcer healed, no muscularis mucosa	##	28	43.3
32	H.I.	53 m	〃	#	thin, metaplasia	hypertrophic changes	medium (2×2.8×1) (2×3×1) kissing ulcer		30	33.1
33	T.K.	43 f	〃	#	metaplasia		big (6×3×3), to muscularis, muscularis, mucosa turned down	#		30
34	K.Y.	60 m	〃	#	superficial necrosis, metaplasia	replaced by conn. tiss. in deep layer	small (3×2×2), to subserosa, muscularis mucosa turned down, defect of proper muscular	#		9.7
35	T.O.	39 f	〃	+	metaplasia	conn. tiss. proliferated, muscularis cut	medium (5.5×2.5×2), to serosa, muscularis turned up and down, superficial necrosis	##	78	32.4
36	N.K.	44 f	〃	#	metaplasia, glands disappeared		small (5×3×1), to muscularis, defect of muscularis in superficial ulcer	–	–	37
37	I.K.	33 m	〃	+	thin	muscularis mucosa was cut	big (12×10×3), to serosa, muscularis turned up and down	##	62	34
38	N.K.	64 m	〃	#		conn. tiss. proliferated	big (6.7×5.2×3), to serosa, muscularis turned down	#		34.5
39	T.K.	71 m	〃	##	thin, metaplasia, glands disappeared		big (7.6×3.5×3), to serosa, muscularis turned up and down	–		26.8
40	S.S.	59 m	〃	##	thin, metaplasia	conn. tiss. proliferated	medium (5×5×3), to serosa, superficial ulcer	#	60	49.3
41	N.K.	49 f	〃	#	metaplasia, superficial necrosis		small (3×3×3), to subserosa, a few superficial ulcers	#	–	49.2
42	T.A.	56 m	aberrant-cancer	##	thin, glands disappeared		big (8.5×11×4), to serosa, mucosa ulcerous, aberration of accessory pancreas	##	6	12
43	H.G.	45 m	〃	##			big (9×8.5×) to perigastric tiss., mucosal cell vacuous, cholistom?	#		23.9

a) The decrease in catalase activity of liver in relation to the difference in types of gastric cancer is shown in Fig. 1. We found that catalase activity of the liver in the control group was about 37% of the normal under the above described conditions. That is, the liver catalase activity in gastric ulcer patients was: 15.5–49, average 36.1 in 13 cases; in gastritis patients: 10.5–70, average 38.2 in 12 cases. The liver catalase activity in gastric cancer patients was generally lower than in the former (8–49.3, average 28.6 in 43 cases). Catalase activity in gastric cancer supposed to be developed on the gastritis is: 8.6–40.0, average 24.6 in 13 cases; in those developed on a gastric polyp: 8–39.6, average 26.0 in 14 cases. A significant difference was noted between averages of these two groups of gastric cancers and noncancerous controls (gastric ulcers and gastritis). It is noted here, that the liver catalase activity of the ulcer-cancers group (9.7–49.3, average 35.1 in 14 cases) showed no significant difference from the control.

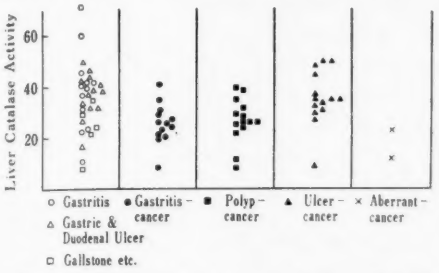

Fig. 1. Decrease in liver catalase activity in relation to different types of gastric cancer.

b) In gastric cancer cases the atrophic changes of the gastric mucosa surrounding the cancerous focus was examined. They can be classified into 3 stages, namely (i) negligible or slight, (ii) mdium, (iii) marked. The relation of decrease in catalase activity in liver slices and the atrophic changes of the mucosa is shown in Fig. 2. The average of catalase activity in group (i) is 48.1, in (ii) 30.8, and in (iii) 21.2. It is noteworthy that the decrease in catalase activity in liver corresponds fairly well with the grade of atrophic changes in the gastric mucosa. It is noted, too, that in most of the gastric cancers supposed to be developed upon deep ulcers, a marked atrophic change in the gastric mucosa is usually not found.

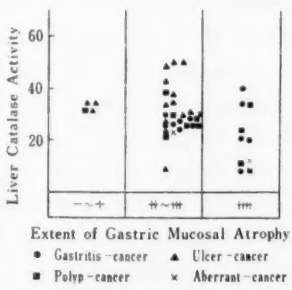

Fig. 2. Relation of decrease in liver catalase activity to the atrophic changes in gastric mucosa.

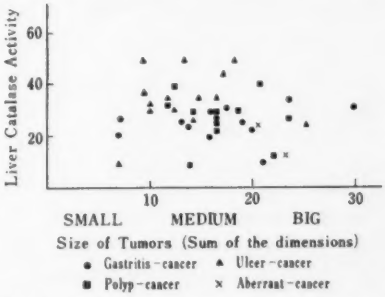

Fig. 3. Relation between size of tumor and liver catalase activity.

c) The relation between the size of the tumor and catalase activity is shown in Fig. 3. No relation could be found between the decrease of catalase activity of the liver and the size of the tumor.

d) No relationship could be found either between the gastric acidity and catalase activity of the liver. (Fig. 4)

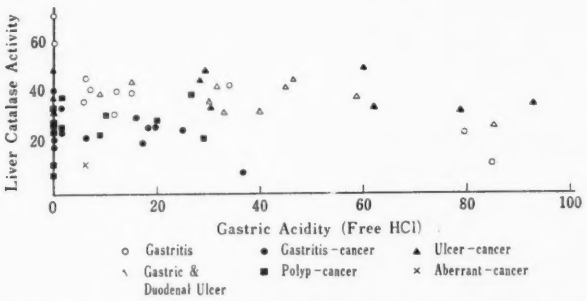

Fig. 4. Relation between gastric acidity and liver catalase activity.

2) ***Histological and histochmical findings on the liver and the hepatic catalase activity :—***

The liver specimens were examined for the following characters: 1) dilatative or restrictive changes in the sinusoids, 2) nature of liver cell traveculae; especially their curvature or position, 3) the regularity of arrangement of the liver cells, 4) thickening or round-cell infiltration of Glisson's sheath, 5) appearance of atypical or degenerated nuclei in liver cells, 6) vacuole formation or concentration in the protoplasm, 7) presence of fatty drops, attitude of glycogen, nucleic acid, and mitochondria. The details of the examination are shown in Table 3.

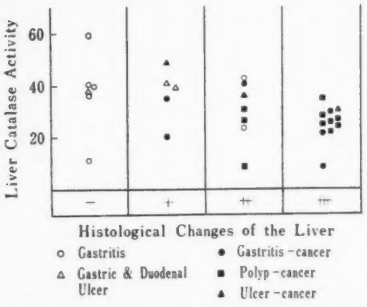

Fig. 5. Relation between histological changes in the liver and catalase activity.

The relation of histological changes in the liver to catalase activity is shown in Fig. 5. Generally speaking, catalase activity decreased in highly affected livers. The histological changes in the liver were classified into 4 groups: (−), (+), (++) and (+++). No significant difference in catalase activity is found between groups (−) and (+), (+) and (++), or (++) and (+++). But between groups (−) and (++), and (+) and (+++), a significant difference is noted. In cancer groups histological changes of the liver were almost always found, but in the non-cancer group such changes were infrequently observed, and if any, were relatively slight.

Table 3. Histological and histochemical findings in the liver in relation to catalase activity

No.	Name	Diagnosis	Liver catalase activity	Changes of liver tissue							
				General structures							
				Sinusoid	Liver cell trabecula	Glisson's sheath	Liver cells	Fat	Glycogen	Nucleic Acids	General condition
1	D.N.	chr. atr. gastritis	40	−	−	−	−	±			−
2	M.J.	〃	39.5	−	−	±	−				−
3	Y.M.	〃	41.3	−	−	−	+	+	positive-cells in the border	−	++
5	M.S.	〃	36	−	−	±	±	+			−
6	U.S.	〃	10.5	−	−	±	−	±	almos none		−
10	F.K.	antrum atrophic gastritis	59			±	±	±		reticular in the center	−
11	H.G.	〃	23	+	−		±	+	many positive-cells	reticular in all the layer	++
13	T.K.	gastric ulcer & antrum gastritis	40	±	±	+					+
14	A.K.	〃	39	−		+	±	+	marked in the center	diffuse in all the layer	+
18	F.J.	gastric ulcer	37.5	−	±	−	−				−
1	Y.M.	gastritis-cancer	40	±	+	+	±				++
2	Y.M.	〃	8.6	+	±	+	+				+++
3	H.R.	〃	20.3	+	+	+	+	+	positive-cells few		+++
4	K.W.	〃	20	±	+	±	−	+			+
5	S.N.	〃	34	±	+	±	−				+
14	S.G.	polyp-cancer	21	+	+	+	+				+++
15	N.K.	〃	34.4	+	+	±	+				+++
16	J.N.	〃	24.6	+	+	+	+	±	medium in all the layer		+++
17	I.M.	〃	8	+	−	+	−	+	few in the border		++
18	T.K.	〃	26	+	+	+	−		〃		+++
19	I.W.	〃	28	+	±	+	++	+	almost none	diffuse	+++
20	Y.M.	〃	23	+	+	+	+	+	〃		+++
21	N.O.	〃	26	+	±	+	±	±	positive-cell in the center	reticular in the center	++
22	M.T.	〃	39.6	+	−	+	−	+			++
23	T.R.	〃	26	+	+	+	−	+	positive-cells in the center	reticular in all the layer	+++
24	N.O.	〃	29		+	+	+	+	positive-cells in all the layer		+++
28	K.S.	ulcer-cancer	34.9	+	+		+				++
29	S.B.	〃	29 5	+	+	±	++				+++
30	S.B.	〃	48	+	±	±	±				+

+and−in this table indicate presence and absence of dilatative or restrictive changes in the sinusoid, of irregularity in the nature of liver cell trabecula, of thickening or round-cell infiltration in Glisson's sheath, of atypical or degenerated nucleus and vacuole formation or concentration in the protoplasm in liver cells, and finally presence and absence of fatty drops.

Discussion

Liver slices were excised from a part where no local disorder such as metastasis or adhesion existed. However, even in the non-cancerous control group the liver could possibly have been affected primarily or secondarily by original diseases. The next problem to be considered is the time between the beginning of laparotomy and the excision of liver. Even though the liver slice was examined immediately after excision, the excision usually took place one hour after the beginning of the laparotomy. In addition patients were always fasted for 12 hours before abdominal operations and this might affect liver function.

However, material both from cancerous and the non-cancerous groups of patients was examined under the same conditions. So, when a considerable difference between the two groups was found it may be regarded as significant.

In the group of patients with gastric cancer originating from deep penetrating ulcers, the liver catalase activity was relatively high, being almost the same as in the control group. In this group of gastric cancer, a regressive change diffusely in the gastric mucosa was seldom observed and if any, the extent of the change was slight. On the contrary, in diffuse infiltrating cancers such as type III and IV of Borrmann's classification, a remarkable decrease in liver catalase activity was almost always observed. Correspondingly, a diffuse regressive change was usually noted in most of the gastric mucosa. These facts suggest the possibility of quantitative or qualitative changes in the enzymes containing iron, such as catalase, in cases of advanced regressive change in the gastric mucosa. Regulation of iron absorption by the specific function of gastric and duodenal mucosal cells has already been pointed out by Granick (14) and others (15–18), and decrease of liver catalase activity resulting from an iron deficient diet has been demonstrated by Schultze (23). When we examined gastric acidity, however, we found that it had no relation to the liver catalase activity. Appleman et al. (13) stated that decrease of liver catalase activity was often observed in animals in which swelling of the liver and spleen was confirmed. We have also found a remarkable decrease in liver catalase activity in a group of gastric cancer patients in which a marked regressive change was found in the liver. However, in cancer patients in which histological changes in the liver were moderate, a marked decrease of catalase activity was observed, while in non-cancerous patients, if there were only moderate changes in the liver, the catalase activity of liver was normal. These results show that there is little relation between the decrease of the hepatic catalase activity and the histological changes of the liver. This is also in accordance with the opinion of Greenstein and others who emphasized the specific decrease of the liver catalase activity in relation to the growth of a neoplasm. Malnutrition in cancer

patients may be related to this, but cannot by itself explain the conspicuous decrease of liver catalase activity as demonstrated by experimental work of Weil-Malherbe and Schade (11). Formerly Begg et al. (24) attributed the decrease of liver catalase activity in cancer patients to dysfunction of the adrenal cortex. However, Begg (25) later withdrew this opinion since he found that the phenomenon was non-specific. On this point our results supports the report of Mider et al. (29) who observed a remarkable decrease of the liver catalase activity at the initial stage of cancer development. They postulated the presence of a metabolic disorder, specific to the tumor, which preceds the morphological changes in the liver.

Summary

Small liver slices were taken from patients during gastric operations and the catalase activity of the liver was estimated. In gastric cancer a decrease of the catalase activity in liver was generally noted, particularly in those cases in which diffuse atrophic changes in the gastric mucosa were present.

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